EFFECTS OF DOMESTIC FREEZING TEMPERATURE (-20˚C) AND BLANCHING ON THE FUNCTIONAL PROPERTIES AND CONSTITUENTS OF THREE COMMON CULINARY HERBS OF LAMIACEAE FAMILY.

A DISSERTATION PRESENTED

BY

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SCHOOL OF LIFE SCIENCES

DEDICATION

Dedicated to Almighty God and my family

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ABSTRACT

The effects of blanching prior to freezing on the functional constituents (ascorbic acid, selenium, phytic acid and phenolic content) and properties (total antioxidant activity, enzyme inhibition activity and prebiotic functions) of 3 well known *Lamiceae* herbs (mint/*Mentha piperita*, thyme/*Thymus vulgaris* and basil/O*cmium basicilla*) were investigated. Comparisons were made between herbs and across different treatments (fresh, un-blanched frozen (-20˚C) and blanched frozen). Due to the complexity of antioxidant and phenolic compounds/components of plants, extracts for total phenolics and antioxidant activities were made in methanol and water.

Generally, among all assayed herbs, mint showed superiority compared to other herbs in terms of functional constituents and properties. However, results of antioxidant capacity/content (DPPH, ORAC, FRAP and CUPRAC) and TPC varied inconsistently across herbs, treatments and assays.

Results also showed that frozen herbs have lower selenium, ascorbic (total, and reduced) and phytic acid content compared to fresh herbs. Furthermore, results of ascorbic acid content showed a significantly lower dehydroascorbic acid (DHA) content in frozen herbs than fresh herbs.

Results of enzyme inhibition showed a moderate to very low α-amylase inhibition ability by all herbs which further reduced on freezing and blanching. Furthermore, compared to other herbs, mint showed the highest α-glucosidase inhibition which reduced on freezing and blanching. Lineweaver-Burke plot showed that fresh and un-balnched frozen mint displayed un-competetive mode of inhibition while blanched frozen mint showed mixed inhibition. Freezing tended to improve the α-glucosidase inhibition effects of thyme and basil which was not evident in fresh extracts. None of the herbs showed inhibitory effects against ACE.

All herbs showed significant prebiotic effects on probiotic bacteria *L. rhamnosus* and *B.bifidum.* However, effects of freezing and blanching varied inconsistently among herbs

Results of the functional properties correlated with individual phenolic acid content of herbs. This therefore shows that the product of the hydrolysis of these individual phenolic compounds play significant role in the functional properties of the assayed herbs.

The findings from this research have therefore shown that freezing (blanching or without blanching) can positively or negatively affect the assayed functional constituents and properties

of assayed *lamiceae* herbs. However, in some instances, there is no difference between the functional constituents of fresh and frozen (un-blanched and blanched).

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1.1 LITERATURE REVIEW

The current focus of Nutritionists is on achieving optimum nutrition, improving the quality of life and achieving optimum life expectancy. This is achieved by identifying food ingredients or compounds which when added to a balanced meal improve its capacity to fight diseases and promote good health (Glenn, et al., 2000). The outcome of this is the concept of functional foods.

A food or food component is regarded as "functional" if it satisfactorily demonstrates its ability to beneficially influence one or more target functions in the body beyond its adequate nutritional value, in such a way that it reduces the risk of disease or improves health and wellbeing (Glenn, et al., 2000).

1.2 FUNCTIONAL COMPOUNDS OF PLANTS AND THEIR FUNCTIONAL EFFECTS

There are several functional compounds which are produced within plants besides the primary biosynthetic and metabolic routes of compounds aimed at plant growth and development. The most studied includes Vitamins C and E, folates and phenolic compounds and their oils (Glenn, et al., 2000). Some of the compounds may be specific to a particular plant while others are widely present in the majority of plant species. This thesis is focused on the phenolic compounds, selenium, phytic and ascorbic acid.

1.2.1 PHENOLIC COMPOUNDS

Phenolic compounds are one of the most studied bioactive compounds of herbs (Peter, 2006). The role of phenolic compounds as protective dietary constituents has become an increasingly important area of human nutrition research. Phenolic compounds accumulate in relatively high amounts in plants and appear to have innumerable supplemental functions in a plant's life cycle (Crozier *et al*., 2006). However, long term intakes may display a potential for modulating human metabolism in a manner favourable for the prevention or reduction of degenerative diseases caused by oxidative stress such as cancer, diabetes, obesity and cardiovascular diseases (Riboli and Norat, 2003). The protective effects of phenolic compounds are thought to be due to direct scavenging of free radicals (Heim *et al*., 2002). Furthermore, phenolic compounds are known to have multi-functional properties such as reducing agents, hydrogen

donation and acting as singlet oxygen quenchers. However most importantly they have a capacity to act as antioxidants protecting the body from reactive oxygen and nitrogen species (Shahidi and Naczk, 1995); as well as having been associated with therapeutic effects including antihypertensive and anti-diabetic treatments (Kwon, *et al.* 2006, Vattem, *et al*. 2005) and antimicrobial properties (Shetty, 2001; Shetty *et al*, 2005).

There are over eight thousand recorded naturally occurring plant phenols which contain at least one aromatic ring with one or more attached –OH groups, in addition to another substituent (Balasundram, *et al* 2006). These are further divided into 15 major structural classes characterised by their carbon skeleton (Yang & Guido, 2016). These include: C6, simple phenols (resorcinol); C6-C1, phenolic acids (*p* hydroxybenzoic acid); C6-C2, acetophenones and phenylacetic acids; C6-C3, hydroxycinnamic acids (caffeic acid); C6-C4, hydroxyanthraquinones (physcion); C6-C2-C6, stilbenes (resveratrol); C6-C3-C6, flavonoids (quercetin); (C6-C3)2, lignans (matairesinol); (C6-C3- C6)2, biflavonoids (agathisflavone); (C6-C3)n, lignins; (C6-C3-C6)n, condensed tannins (procyanidin) (Apak, *et al.* 2007).
Each of these classes of phenolic compounds have individual effects on the plant and important nutritional benefits when ingested.

1.2.1.1 FLAVONOIDS

Flavonoids are polyphenolic compounds comprising of 15 carbons and 2 aromatic rings connected by a 3-carbon bridge. They can be divided into different structural classes based on modifications of the central C-ring. They include flavanols, flavones, flavan-3-ols, flavanones, isoflavones and anthocyanidins.
Flavanoids are widespread in foods and beverages such as fruits, vegetables, chocolate, teas and wines. They are found in most plants and give rise to coloured compounds such as anthocyanins. They are however regarded as the predominant phenolics in plant foods (Falcon Ferreyra, et al., 2012) and account for about two-thirds of the dietary phenols (Scalbert 2000).They act as antioxidants, although one of their main functions is to protect plants from ultra violet radiation and intense light (Glenn, et al., 2000).
Flavonols are the most prevalent of all the flavonoids in plant materials. They include quercetin, kaemferol and myricetin with quercetin seen as the most ubiquitous. In addition to their antioxidant effects, flavonols have been shown to interfere with several physiological and pathological processes. These include the inhibition of low density lipolipid oxidation,

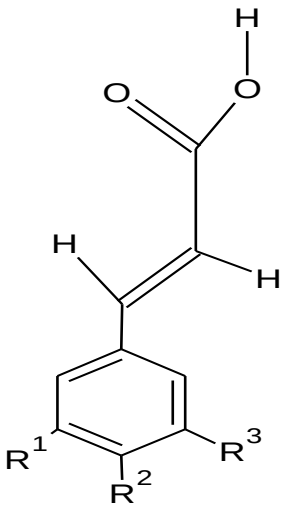
reduction of adhesion molecules and other inflammatory markers, hence their usefulness in prevention of inflammatory damage (Perez-Vizcaino, 2010) .

1.2.1.2 PHENOLIC ACIDS

Predominant phenolic acids of plant origin include the hydroxycinnamates (C_6-C_3) and hydroxybenzoates (C_6-C_1) derivatives (Clifford, 2000; Tomas-Barberan and Clifford, 2000). The content of hydroxybenzoic acid in plants is lower than hydroxycinnamic acids (Widhalm & Dudareva, 2015). The hydroxybenzoates (Figure 1.1b) are commonly present as gallic acid, p-hydroxybenzoic acid, protocatechuic, syringic and vanillic acids. Gallic acid is the base unit of gallotannins and is usually present in bound form. They are known to be components of complex structures like lignins and hydrolysable tannins and are usually found in plant foods as derivatives of sugars and organic acids. Benzoic acid-4-*O*-glucosde is common phenolic found in herbs like parsley (*petroselinum crispium)* and dill (*Anethum graveolens*) (Crozier *et al*., 2006).

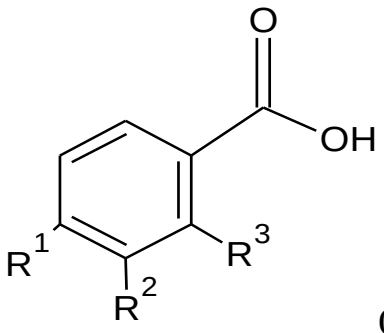
Hydroxycinammates (Figure 1.1a) include p-coumeric, caffeic and ferrulic acids and frequently accumulate as their respective tartarate esters of coutaric, caftairic and fertaric acids. Hydroxycinnamic acids are present in a wide range of berries, fruits and beverages (Mattila *et al*., 2006) and also in many vegetables (Shahidi & Chandrasekara, 2010) and cereals (Kern et al., 2003). Conjugates of caffeic acid are found in fruits and vegetables inform of chlorogenic (Figure 1.2) and quinic acid (Crozier *et al*., 2006). Quinic acid can be conjugated as mono-, di-, tri-, and tetra (Clifford, 2000).

These phenolic acids differ in patterns of hydroxylations and methoxylations of their aromatic rings (Prior, *et al*, 2006)



(a) General structure of hydrocinnamic acid

	R^1	R^2	R^3
p-coumeric	**H**	**OH**	**H**
Caffeic acid	**H**	**OH**	**OH**
Ferrulic	**CH_3 O**	**OH**	**H**
Sinapic	**CH_3 O**	**OH**	**CH_3 O**



(b) General structure of hydrobenzoic acid

	R^1	R^2	R^3
p-hydrozybenzoic acid	**H**	**OH**	**H**
Protocatechuic acid	**H**	**OH**	**OH**
Vanillic	**CH_3 O**	**OH**	**H**
Syringic	**CH_3 O**	**OH**	**CH_3 O**
Gallic acid	**OH**	**OH**	**OH**

Figure 1.1 General chemical structures of hydroxycinnamic and hydroxybenzoic acids derivatives

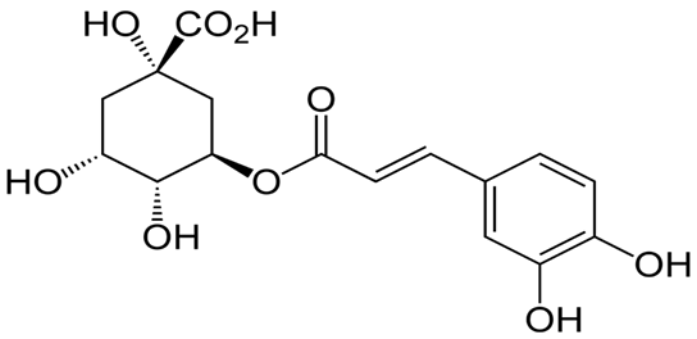


Figure 1.2 Chemical structure of chlorogenic acid (5- *O* -caffeoylquinic acid)

Phenolic acids have received considerable attention as potentially protective factors against several health disorders (cancer, type II diabetes mellitus and coronary heart diseases), partly due to their antioxidant potency and their ubiquity in a wide range of commonly consumed plant product (Manach, et al, 2004).

1.2.1.3 DIETARY INTAKE/SOURCES OF PHENOLICS

Plant phenolics form an integral part of our diets. Phenolics are widely distributed in the plant kingdom and cover a wide range of classes with interesting beneficial health effects. The most common phenolics are found in fruits and vegetables include flavonols, anthocyanins, flavan-3-ols 9catechins) and hydroxycinnamates (Glenn, et al., 2000).Common rich sources of phenolics include cereals, soybeans, tea, coffee, legumes, fruits and herbs (Spencer, et al., 2008).

1.2.2 PHYTIC ACID

Phytic acid (myoinositol hexa-phosphoric acid, IP6) is the major phosphorus storage compound of most seeds and cereal grains, it may account for more than 70% of the total phosphorus. It forms 1–5% by weight of edible legumes, cereals, oil seeds, pollens and nuts (Das, et al., 2012). Excess phytic acid has a strong ability to chelate multivalent metal ions, such as copper, zinc, calcium and iron at physiological pH leading to the formation of insoluble complexes (Gupta, et al., 2015). Its ability to form stable complexes with multivalent cations is due to its unique structure (Figure 1.3) of 12 replaceable protons and high density of negatively charged phosphate groups.

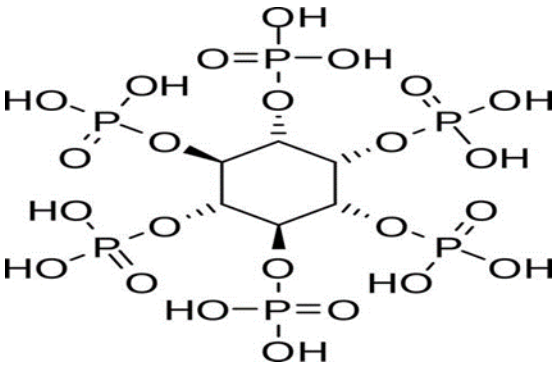


Figure 1.1 Chemical structure of phytic acid

Phytic acid consequently reduces the bioavailability of these minerals which are essential for human nutrition and health, making them unavailable for intestinal absorption and for their respective functions (Gupta, et al., 2015). Hence it was regarded as an anti-nutritional compound (Bohn *et al*., 2007; Li *et al*., 2008; Schelemmer *et al.*, 2009).

However, several health benefits have been associated with phytic acid intake. These include the reduction of hepatic lipid level associated with a reduction in fatty liver disease (Onomi *et al* 2004) and chelation of copper thereby preventing the interaction of copper with biomolecules, lowering the generation of reactive oxygen species (ROS).

Furthermore, the formation of complexes with Fe^{2+} ions has been associated with a favourable reduction in the formation of hydroxyl radicals in the colon (Graf and Eaton, 1993). Its reaction with iron leads to the formation of iron-phytate chelate which is totally inert in the Fenton reaction. The ability of phytic acid to form this complex makes Fe unavailable for hydroxyl radical formation, a reaction which exhibits its ability to act as an antioxidant. Due to its antioxidative potential, it has aroused great interest as a potential food preservative and therapy for pathological diseases caused by free radicals (Soares *et al*., 2004; Stodolak, *et al*., 2007; Harbach *et al*., 2007). As a preservative it has been found to inhibit lipid peroxidation in beef, thereby inhibiting the formation of metmyoglobin which is responsible for the brown discolouration of meat (Bozena, *et al*., 2007).

Its chelating ability has been suggested to be beneficial to human beings through lowering serum cholesterol and triglyceride and the suppression of Fe mediated oxidation (Lee, *et al*., 2005). Furthermore, research suggests that it may inhibit the development of renal stones (Dost

and Tokul, 2006) caused by kidney calcification. Kidney calcification is caused by consuming diets poor in whole products and rich in calcium. Therefore, the consumption of phytic acid rich diets (legumes, whole grain foods, etc) has been said to maintain calcium urinary levels which inhibits calcium oxalate crystallization *in vitro* hence preventing renal stone formation (Grases, *et al*., 2000).

Sources of phytic acid in food include legumes, cereals, nuts (cashew nuts, walnuts and almonds) and oilseeds. However, the phytic acid content in whole seeds and bran rich grains are higher than that of refined/polished seeds or refined grains (Lestienne *et al*, 2005, Schlemmer *et al*, 2009). This is because the phytic acid content is drastically reduced during processing methods such as soaking and cooking (Vellingiri and Hans, 2010). Its content in culinary herbs has not been reported.

1.2.3 SELENIUM

Selenium is regarded as an essential nutrient for humans because it is an essential component of several major metabolic pathways, including thyroid hormone metabolism, antioxidant defence systems and immune function. It has been recognised as an integral component of different enzymes such as thioredoxin reductase and glutathione peroxidase, which participate in the antioxidant protection of cells (Birringa, *et al*., 2002).

The glutathione peroxidise is one of the antioxidants for the body which catalyses several reactions and protects organisms from oxidative damage by reducing lipid peroxide and free hydroperoxide to their corresponding alcohols and water respectively. Furthermore, it inhibits the toxicity of some metals such as lead, mercury and cadmium (Klapec *et al*., 2004) and reduces risk of cancer (Tinggi, 2003). Selenium content of plants varies from species to species. This is because most plants are not able to accumulate selenium while others (such as sulphur containing plants) do. Deprivation of selenium is associated with reduced antioxidant protection, redox regulation and energy production while toxicity can lead to adverse effect referred to as selenosis (Combi, 2001; Thomas, 2004). Furthermore, selenium has been reported as to act as a prebiotic to probiotic bacteria in healthy human guts (Molan *et al*, 2009).

Selenium is a metalloid and can be present in organic forms as selenoproteins such as selenomethionine (Se_{Meth}), selenocycteine (Se_{Cys}), cellular or classical glutathione peroxidase, plasma glutathione peroxidise, phospholipid hydroperoxide glutathione, gastrointestinal

glutathione peroxidise, selenoprotein P, iodothyronine deiodinase, selenoprotein W, thioredoxin reductase and selenophosphate synthase. It can also be present in inorganic forms as selenite and selenate (Arner, 2011).

Selenoproteins such as thioredoxin reductase and glutathione peroxidise are the only functional selenium in mammalian system. These enzymes catalyse the destruction of hydrogen peroxide or lipid hydroperoxides by providing reducing power and catalysing several biochemical processes such as the reduction of peroxides subsequently defending against oxidative stress and cellular damage. Another important metabolite of selenium is the tetraiodothyronine (T_4) which is essential in the conversion of thyroxin to its physiologically active form triiodothyronine (T_3) (Mehdi, et al., 2013). Thyroid deiodinases aid in the formation and regulation of thyroid hormone while selenoproteins P and W play important roles in oxidant defence and metabolism in plasma and muscles respectively. Sperm mitochondrial capsule selenoprotein (Phospholipid GSHPx) is distributed in the sperm tail is important for sperm flagella and as a consequence improves sperm motility. Furthermore, GSHPx helps in the structure of mature sperm and protects developing sperm from oxidative damage (Razaeian, et al., 2016).

Generally, it has been reported that selenium (Se) may reduce the incidence of cancer and its associated diseases in humans (Finley *et al*., 2001; Kolachi *et al*., 2010). Furthermore, Molan, *et al*., (2009) have also reported the prebiotic function of selenium-enriched tea on gut probiotic bacteria.

1.2.3.1 DIETARY SOURCES AND BIOAVAILABILITY OF SELENIUM

The selenium content of foods varies geographically and is dependent on several factors including the selenium content of the soil or environment the plant is grown in. The selenium content of any food material therefore depends on the availability of external selenium and the plants ability to accumulate selenium (Dumont *et al*., 2006). However, plants such as some mushrooms, garlic (*Allium sativum*) and canola (*Brassica napus*) have been found to be selenium accumulators of up to >1000mg Se/kg without exhibiting any negative effects (Dumont *et al*., 2006). This is because, of the reduction of the intracellular Se concentration of selenocysteine (Se_{Cys}) and selenomethionine (Se_{Meth}) which are normally incorporated into proteins (Dumont *et al.*, 2006). Hence appropriate consumption can make these plants good source of dietary selenium.

Selenium is sometimes used in the food industries in the form of inorganic selenium (selenite and selenite) as supplements or to fortify food products (Dumont *et al.*, 2006). However, they are not regarded as major dietary source of selenium (Vonderheide *et al*., 2002).

1.2.4 ASCORBIC ACID

Ascorbic acid (also known as vitamin C) is a naturally occurring organic compound derived from glucose which forms one of the most important molecules in the human diet. It is one of the most important water-soluble vitamins found in most fruits, vegetables and herbs. Ascorbic acid can be synthesized *de novo* in the hexuronic acid pathway of the liver or the kidney of species having L-gulono-1,4-lactone oxidase activity. However, human beings can't synthesis ascorbic acid because the gene encoding the enzyme catalyzing the last step in its biosynthesis (L-gulono-1-4-lactone oxidase) is non-functional (Radzio *et al.*, 2003). Humans therefore depend solely on plant sources such as fruits and vegetables. It plays several important roles in living organisms and plants such as an antioxidant, an enzyme co-factor, has a role in cell wall expansion and metabolism and also helps during photosynthesis (Ivanov, 2014). These roles of ascorbic acid have been linked to its ability to act as a reversible biological reductant (Senapati, et al., 2012). As an antioxidant it has been found to help neutralize most relevant reactive oxygen and nitrogen species (Nimse & Pal, 2015), suppress cyclophosphamide induced lipid peroxidation (Supratim, *et al*., 2005), reducing gastric cancer by trapping nitrogen (Zhang, et al., 2002). It is also known for its ability to synthesize collagen which prevents scurvy.

Ascorbic acid has been reported to have the ability to regenerate some biologically important antioxidants, like glutathione and vitamin E, into their reduced state (Ivanov, 2014). As a strong electron donor, it provides intra and extra cellular reducing power for a variety of biochemical reactions. Among other antioxidative functions, it has been reported to protect the lens and retina against damage caused by UV (Preedy, 2014), prevent plasma and low density lipoprotein oxidation (Shariat, et al., 2013). The daily recommended dose of ascorbic acid is quite high and varies by gender, age, lifestyle and health conditions of individuals and ranges from 20- 200mg/day.

Ascorbic acid is found in plant tissues; however, its availability and content vary from plant species/cultivars. The amount of ascorbic acid present in plants is widely distributed in the

cytosol, chloroplast, vacuoles, mitochondria and cell wall and are said to be more than their chlorophyll content and is said to be dependent of the maturity, weather and processing conditions and the varieties of plants (Ivanov, 2014).

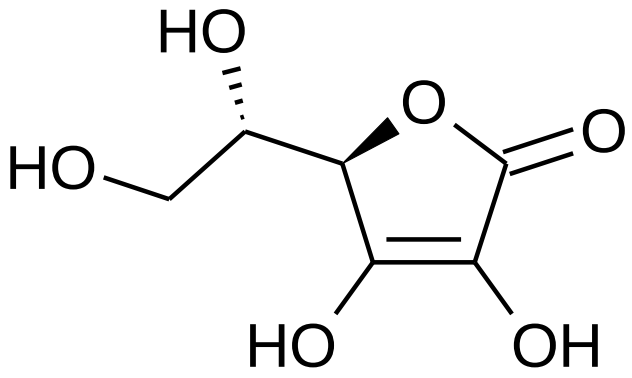


Figure 1.2 Chemical structure of ascorbic acid

1.3 BIOSYNTHESIS OF FUNCTIONAL COMPOUNDS IN PLANTS

Plants synthesize and accumulate large number of bioactive compounds. Most functional phytochemicals of interest are synthesised in plants either by the biosynthetic pathway (Phenylpropanoid) or the isoprenoid pathway (Glenn, et al., 2000). The phenylpropanoid pathway leads to the production of lignins and their phenolic ester precursors, the flavones and related compounds, and isoflavones (Vogt, 2016). The isoprenoid pathway leads to the formation of terpenes, sterols, carotenoids and tocopherols (Paddon & Keasling, 2014). The biosynthesis of some functional compounds of interest to this thesis is briefly discussed below.

1.3.1 BIOSYNTHESIS OF PHENOLIC COMPOUNDS IN PLANTS

Plants contain a large variety of phenolics which include simple phenolics, phenolic acids, flavonoids, tannins and lignins. There are about 8,000 known structures of plant phenols which account for about 40% of organic carbon circulating in the biosphere. Plant phenols, among numerous other functions, are responsible for the protection of plants, vascular tissue structure, the flavour and colours of plants. The majority of plant phenols are formed via the shikimate/arogenate pathway (Figure 1.5). This pathway is known to lead to the formation of three aromatic amino acids; L-phenylalamine, L-tyrosine and L-tryptophan.

These biosynthetic pathways represent a complex biological regulatory system which takes place in vascular plants and has been reported to be vital for their growth, development, and survival (Costa *et al.*, 2003). The shikimate pathway links metabolism of carbohydrates to

biosynthesis of lignins and their phenolic ester precursors, the flavones, isoflavones and related compounds (Glenn, et al., 2000). In a sequence of seven metabolic steps, phosphoenolpyruvate and erythrose 4-phosphate are converted to chorismate, the precursor of the aromatic amino acids and many aromatic secondary metabolites.

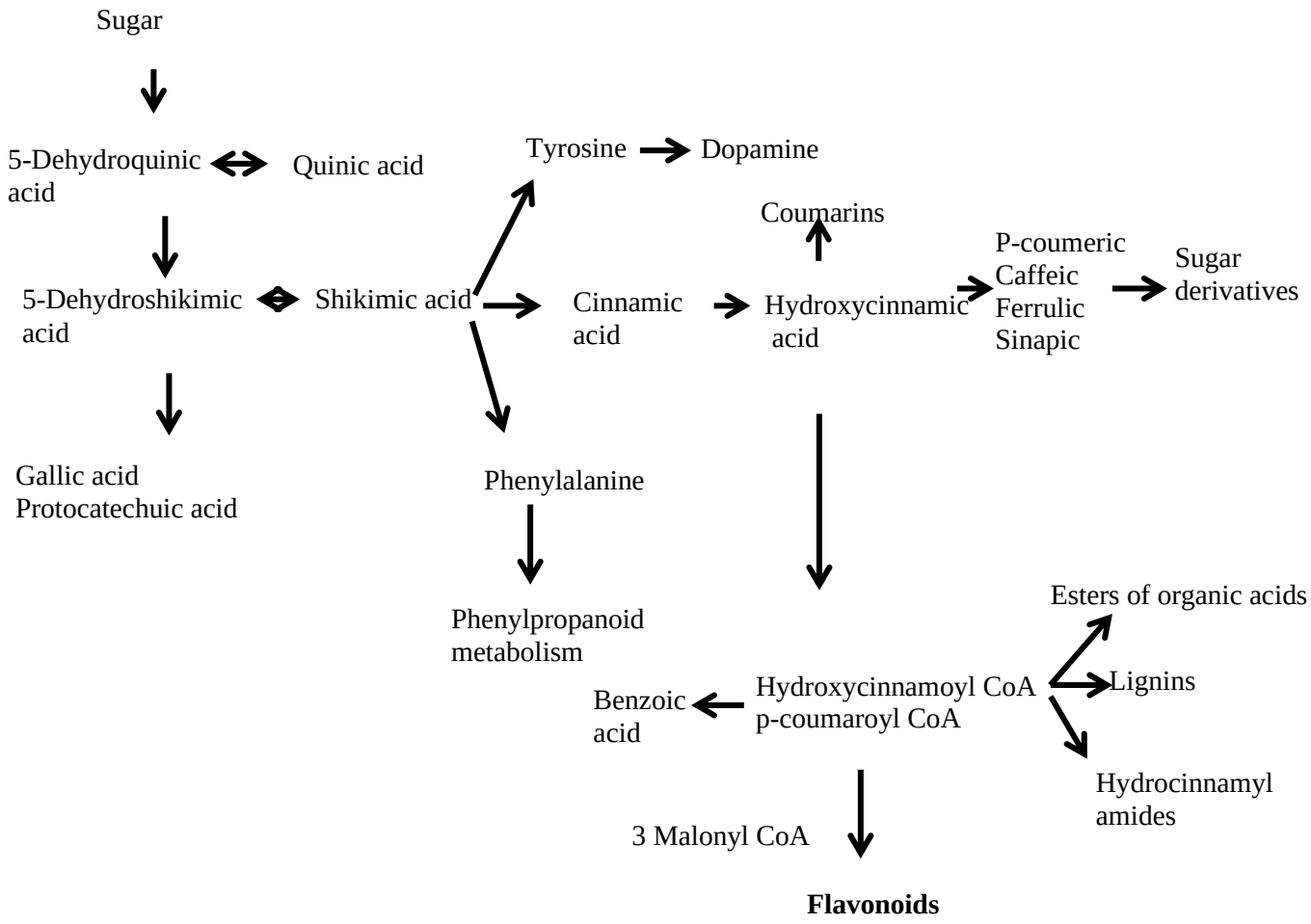


Figure 1.3 General schematic representation of phenolic compound metabolism (modified from Lin et al., 2010).

However, only a few phenolic acids occur in free form. They mostly occur in insoluble/bound forms and have been demonstrated to possess a significantly greater antioxidant activity compared to free and soluble conjugated phenolics (Chandrasekara and Shahidi, 2010; Liyana-Pathirana and Shahidi, 2006). Insoluble/bound phenolic acids are covalently bound to cell wall structural components such as cellulose, hemicelluloses, lignin, pectin and rod-shaped

structural proteins (Wong, 2006). Phenolic acids such as hydroxybenzoic and hydroxycinnamic acids form ether linkages with lignin through their hydroxyl groups in the aromatic ring and ester linkages with structural carbohydrates and proteins through carboxylic group (Bhanja *et al*., 2009; Liyana-Pathirana and Shahidi, 2006). These linkages are said to be responsible for the formation of vast array of derivatives and hence the major factors in the complexity of the analysis of phenolic acid (Robbins, 2003). Bound hydroxycinnamic acids are found to be esters of hydroxyacids such as quinic, shikimic and tartaric acid and their sugar moieties.

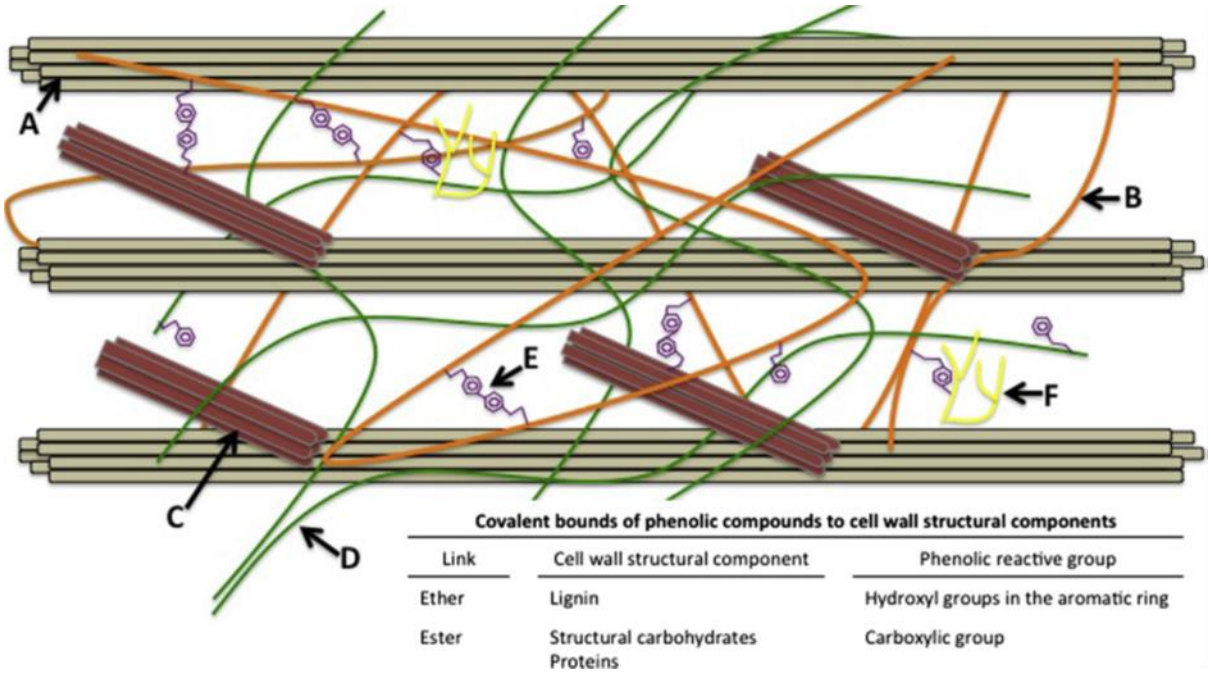


Figure 1.4 Representation of primary cell wall structure of plant material and cross-linking between structural component and phenolic compounds (A) cellulose, (B) Hemicellulose, (C) structural proteins (D) Pectin (E) Phenolic acids (F) Lignin (Acosta-Estrada *et al*., 2014)

Bound forms of phenolic acids can however be released through several food processing methods such as fermentation, extrusion, cooking and alkaline hydrolysis. Fermentation has been reported to increase the total free phenolic acid and total antioxidant activity (Bhanja *et al*., 2009). Thermoplastic extrusion of cereals was reported to release bound phenolics due to breaking of conjugated moieties (Rochin-Medina *et al*., 2012). Alkaline treatments of food are also known to release bound phenolics. Other methods employed to liberate bound phenolic compounds include extraction using different organic solvents including alcohols (methanol

and ethanol), acetone, diethyl ether and ethyl acetate (Pozo-Insfran *et al*., 2006; Gutierrez-Uribe *et al*., 2010)

1.3.2 BIOSYNTHESIS OF ASCORBIC ACID

Ascorbic acid is usually known as the generic term for all the compounds exhibiting biological activity of L-ascorbic acid (Ainsworth & Gillespie, 2007). Hence Ascorbic acid is referred most of the time as total ascorbic acid (TAA).

Ascorbic acid is present as reduced ascorbic acid (AA) and oxidized dehydroascorbic acid (DHA). AA is known as the most biologically active L-ascorbic acid. The AA/DHA ratio can be an indicator of the redox state of a system (Ainsworth & Gillespie, 2007). Furthermore, there is very little information about the contribution of ascorbic acid degradation to the control of ascorbic acid content of plant tissues particularly under environmental stress conditions. Many researchers have also not considered DHA when estimating the ascorbic acid activity in a component. Consequently, it is necessary to assay both reduced and oxidized ascorbic acid content of samples with the total ascorbic acid as the sum of the reduced ascorbic acid (AA) and the oxidized ascorbic acid (DHA).

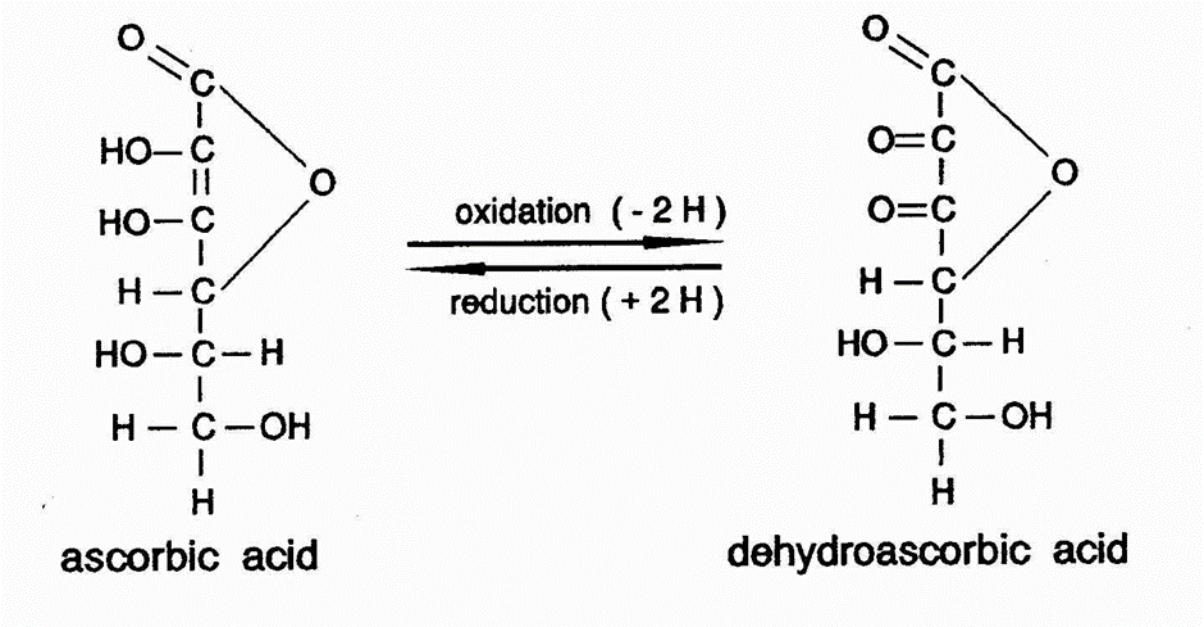


Figure 1.5 Representation of dehydroascorbic acid synthesis

1.3.2.1. REDUCED ASCORBIC ACID

Ascorbic acids in plants are mainly in reduced form (Conklin and Barth, 2003), however they can be oxidized by ascorbic acid oxidase to monodehydroascorbic acid (MDHA) which on further oxidation can form an uncharged molecule dehydroascorbic acid (DHA). The role of AA in metabolism is complicated and its action in protecting against the oxidizing effect of free radicals is of vital importance. AA has been reported to be a cofactor for the activity of dopamine β-hydrolase, the only enzyme involved in the synthesis of small-molecule neurotransmitters, regulate the synthesis of collagen as well as management of cancer (Odriozola-Serrano, et al., 2007)

1.3.2.2 DEHYDROASCORBIC ACID

Oxidation reactions may be induced by increased temperature, high pH, light, presence of oxygen or metals and enzymatic action (Novakova, *et al.*, 2008) giving rise to oxidized ascorbic acid or dehydroascorbic acid (DHA). DHA also possess biological activity of L-ascorbic acid. Although a form of ascorbic acid, DHA does not have antioxidant activity to quench free radicals unless it is reduced emzymatically to AA. It has been reported to have antiviral effects (Kim, et al., 2013) and used as a cure for gingivitis (Anon., 2014). DHA can be enzymatically converted back to AA by DHA reductase, however, if further oxidation occurs, an irreversible diketogluconic acid is formed (Zhang, 2012).

In many horticultural crops, DHA has been reported to represent less than 10% of total ascorbic acid. However, the DHA content of crops is predisposed to increase during storage (Ainsworth & Gillespie, 2007).

1.3.3 BIOSYNTHESIS OF PHYTIC ACID

In plants, phytic acid is synthesised and accumulated in seeds as a phosphorus reservoir. There are several investigations and reports on the synthesis of phytic acid. The early step of phytic acid synthesis which is catalysed by Ins(3)P_1 *myo*-inositol monophosphate synthase (MIPS) is the conversion of D-glucose-6-P to 1L-*myo*-inositol-1-P (Glu-6-P to Ins(3)P_1). The final steps are consistent with sequential phosphorylations of soluble inositol phosphates catalysed by several kinases and phospholipase C-dependent conversion of phosphatidyl inositol phosphate intermediates to Ins(1,4,5)P_3 (Loewus and Murthy, 2000; Raboy, 2003).

MIPS activity has been shown to be widely distributed in intracellular compartment such as membrane-bound organelles, cell walls and cytoplasm (Lackey *et al*., 2003). However relatively little is known about the site of phytic acid synthesis and its transportation to vacuoles of seeds.

1.4 FACTORS AFFECTING THE FUNCTIONAL CONSTITUENTS OF PLANTS

There is limited published systematic examination on factors affecting many functional compounds and bioactive phytochemicals of plants. Although the levels of phytochemicals can be greatly influenced by agricultural practices, postharvest practices such as processing and preservative methods have been reported to have great influence on the levels of phytochemicals in plants (Glenn, et al., 2000).

Certain processing and storage methods are said to bring about biochemical changes and other physical alterations which may affect the quality of plant material (Peter, 2006). These changes include appearance and alterations in aroma caused by losses in volatile compounds or the formation of new volatiles as a result of oxidation reactions or esterification reactions (Di Cesare *et al*., 2003; Diaz-Maroto *et al*., 2002).

Processed foods are generally expected to have lower health protecting abilities than fresh ones. This is based on the selected constituents that are deemed indicators of processing damage such as vitamins (Glenn, et al., 2000).

One of the most used preservative methods for fresh herbs and several other plants is freezing. Since the inception of freezing in 1930s, varieties of frozen vegetables have flooded the supermarkets. Freezing is known to delay spoilage and keeps foods safe by preventing microorganisms from growing and by slowing down enzyme activity that causes food to spoil. During freezing, water forms ice crystals thereby making water unavailable for microbial activity. In normal home freezing, the temperature of freezer compartment is usually maintained at ≤-18˚C (Fellows, 2000). However, freezing is not enough to fully stop enzymatic reactions, senescence, and microbial growth, hence blanching must be performed first (Canet, 2004).

Blanching involves a short- term exposure of vegetables to a heat treatment in a water at 85 - 100 ˚C (Teresa Mazzeo, 2015). This treatment has been reported to inactivate enzymatic reactions, hence enhancing safety and some quality attributes (Canet, 2004). However,

blanching has been found to influence the loss of thermal labile compounds such as ascorbic acid (Olivera, 2008).

1.5 EFFECTS OF FREEZING AND BLANCHING ON THE FUNCTIONAL CONSTITUIENTS OF PLANTS

Freezing is recognised as one of the best methods available in the food industry for preserving food products. The decreases in temperature inhibits metabolic processes occurring in the products after harvesting as well as slowing down the rate of microbial growth (Jaiswal, 2012). Frozen vegetables are known to retain their flavour for several months and can be used in the same proportion as their fresh counterparts. Most herbs are frozen into cubes or whole leaves for their easy use and general acceptability for all dishes. Furthermore, frozen vegetables have been reported to retain their colour due to retention of chlorophyll (Pellegrini, et al., 2010). Effects of freezing temperature and the period of freezing on the physio-chemical properties of herbs have also been reported by Hossin *et al*. (2010) and Volden *et al*., (2009). The effects of freezing on some functional compounds of interest is summarised below.

1.5.1 ASCORBIC ACID

Ascorbic is highly susceptible to degradation and oxidation by chemicals and enzymes during processing, cooking and storage of produce (Glenn, et al., 2000). Losses vary with different produce and according to the degree of AA retention; vegetables were classified as high retention (greater than 95% for broccoli, Brussels sprouts); medium retention (65-70% for green pea, spinach, turnip) and low retention (5-30% for asparagus and green beans)

Ascorbic acid oxidase is the enzyme that is directly involved in the loss of ascorbic acid. Other plant enzymes such as phenolase, cytochrome oxidase and peroxidise are indirectly responsible for the loss of ascorbic acid. The loss caused by the action of enzymes can be prevented by blanching and pasteurization. However, some blanching methods are known to retain ascorbic acid more than the others (Jaiswal, 2012). For instance, ascorbic acid was retained more in frozen spinach after steam and microwave steam blanching than with water blanching (Gupta, et al., 2008). However, water blanching is known to ensure more homogenous heat treatment than steam blanching. With steam blanching there is over cooking of produce closer to source of heat (Xiao, et al., 2017).

The first product of ascorbic acid oxidation is dehydroascorbic acid which is the biological active form of ascorbic acid. However, the most nutritionally significant reaction in ascorbic acid degradation is the hydrolytic decomposition of dehydroascorbic acid to for biologically inactive 2,3-diketogluconi acid (Gillespie and Ainsworth, 2007).

1.5.2 PHYTIC ACID

Phytic acid is water soluble and reduction in water may be attributed to leaching out into surrounding water and under concentration gradient which affects the rate of diffusion (Kakati *et al*., 2010). Phytic acid has been reported to be lost during cooking of black gram and reduction by heating was partly attributed to the heat labile nature of phytic acid and formation of insoluble complexes between phytate and other components (Udensi et al., 2007). However, there has been no report on the effect of freezing on the phytic acid content of produce. Hence the information on effects of blanching prior to freezing remains limited to blanching and excluding frozen storage.

1.5.3 SELENIUM

There have been varying and inconsistent reports from different studies on the effects of cooking/processing on selenium content of foods. Some studies have reported that usual cooking procedures do not result in the loss of selenium while some studies reported the volatization of selenium by cooking methods such as boiling, baking and grilling (Dumont *et al*., 2006; Sager, 2006). For instance, Navarro-Alarcon and Lopez-Martinez (2000), reported a 40% loss in selenium content of asparagus and mushrooms when boiled for some minutes. However, the losses reported seemed to vary according to type of food and processes employed. Furthermore, Lu et al (2018), reported a minial loss of 8.1% selenium during the process of steaming, boiling and frying of soybean.

In contrast, other researchers reported an increase in selenium content in all food with cooking, aeration and lyophilisation (Zhang *et al*., 1993). However, no report has been given on the effects of freezing on selenium content of plant materials.

1.5.4 PHENOLICS

There are few reports on the effects of preservation on individual phenolic compounds. However, post harvest operations such as peeling, cutting and slicing have been reported to induce a rapid enzymatic depletion of several naturally occurring polyphenols (McCarthy and

Mattheus, 1994). Furthermore, depending on storage conditions, antioxidant properties of red wine have been reported to increase or decrease (Manzocco *et al*., 1999). Other researchers reported on varying effects of freezing and blanching on herbs and vegetables (Chan, 2014).

1.6 PLANT SOURCES OF FUNCTIONAL COMPOUNDS

Plants have been found to contain many functional compounds which in addition to providing health benefits to human beings and animals also have specific functions within the plants (Peter, 2006). Some functional compounds of plants include vitamins and several secondary metabolites such as phenolic compounds found in fruits, some vegetables and herbs (anthocyanides, flavonoids), organosulphides, lycopene in tomatoes and saponins in soy (Barros *et al*., 2010; Jambor and Czosnowska, 2002; Javanmardi *et al*., 2003, Kwon *et al*., 2006). However, the main focus if this thesis is the functional compounds of herbs.

The most common plant sources of functional compounds include *lamaiceae or labiatae* (rosemary, thyme, basil, mint, oregano, sage, lavender, perilla and savory); *Umbelifereae/Apaiceae* (angelica, anise, arracacha, asafoetida, caraway, carrot, celery, centella, asiatica, chervil, cicely, corrianda/cilantro, cumin, dill, fennel, hemlock, lovage, parsley, parsnip and sea holly) and the *lilaceae/Allium* (chives, garlic). The families of interest in this thesis are the *lamiaceae* family.

Lamiaceae herbs are a large family of chiefly annual or perennial herbs which are grown all over the world. They are herbaceous; rhizome plants that emit quadrangular green or purple stalks. Several species are shrubby or climbing forms or, rarely, small trees. They are especially widely grown in the Mediterranean region, where these plants form a dominant part of the vegetation.

The common *lamiaceae* herbs include rosemary, thyme, basil, mint, oregano, sage, lavender, perilla and savory. The water extract and essential oils of this group of herbs are known to have been used both in the past and present for therapeutic purposes. For instance, the essential oils and other extracts of thyme are used biologically as carminative, antispasmodic, antitussive, expectorant, bactercidal, antihelmintic, and astringent agents as well as in the treatment of dyspepsia, chronic gastritis and diseases of upper respiratory tract (Baranauskiene *et al*., 2003).

Mint apart from containing calcium and phosphorous, is widely used both traditionally and in the pharmaceutical industry for medications including as an antiseptic, anti-asthmatic, stimulative, diaphoretic, stomachic and antispasmodic, in colds, flu, fever, poor digestion, motion sickness, food poisoning, rheumatism, hiccups, stings, ear aches, flatulence and for throat and sinus ailments (Park *et al.*, 2002). Basil has been used in the treatment of headache, cough, diarrhoea and kidney malfunctions (Peter, 2012), insect bites and acne (Waltz, 2012).

These herbs have been reported to be used as anti-cancer therapy by increasing endogenous protective enzymes such as NADPH-oxidase (NOX), protection of DNA from free radical-induced structural damage and encouraging self-destruction of abnormal cells, hence inhibiting the growth of tumours (Hedges and Lister, 2007).

Researchers have reported that the therapeutic effects of *lamaiceae* herbs are due to their content in several phytochemical dominated by the phenolic compounds. (Kwon *et al*, 2006; Vattem *et al*., 2005; Loughrin and Kasperbauer 2001; Tarchoune *et al*., 2009).

1.7. GENERAL HEALTH BENEFITS OF HERBS

The benefits of herbs on human health are often ascribed to their various phytochemicals and their ability to act as antioxidants. However, several other health benefits have recently been linked to the consumption of herbs although the exact mechanism of action is still unclear.

1.7.1. ANTIOXIDANT ACTIVITY AND CONTROL OF OXIDATIVE STRESS

Oxidative stress and antioxidants have become common terms in the discussion of most disease mechanisms. The concept of oxidative stress and antioxidant activity are usually preceded by discussing reactive oxygen and nitrogen species (ROS and RNS), and free radicals.

Reactive oxygen and nitrogen species (ROS and RNS) are an integral part of a normal human metabolism and are contributed by exogenous exposure to oxidizing agents including ionizing radiation, heavy metals, and hypoxia (Jena, 2012). ROS are regarded as the by-products of normal oxygen metabolism in living organisms, and includes singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroperoxyl ($HO_2^{\bullet}$). ROS can be divided into 2 groups, namely, free radicals and non-radicals. Free radicals are molecular species which are capable to exist independently and contains one or more un-paired electron in an atomic orbital, while non-radicals are created when 2 free radicals share their un-

paired electrons. Free radicals can act by either donating or accepting an electron from other molecules, hence acting as either oxidants or reductants (Birben, et al, 2012).

RNS are a family of antimicrobial produced through inducible nitric oxide synthase 2 (NOS2) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. These include nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) (Lovine, et al., 2008).

The overproduction of these ROS/RNS is capable of oxidizing important biomolecules causing potential biological damage referred to as "oxidative stress" (Halliwell and Gutteridge 2007). Oxidative stress occurs in biological systems when there is an overproduction of ROS/RNS on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other. In other words, oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of prooxidant/ antioxidant reactions in living organisms (Ridnor, et. al, 2005).

Oxidative stress may occur in tissues injured by trauma, infection, heat injury, hypertoxia, toxins and excessive exercises. These injured tissues can activate phagocytes, release of free iron, copper ions or disrupt the electron transport chains of oxidative phosphorylation, thereby producing excess ROS (Lobo, et al, 2010). During phagocytosis, cells such as polymorphonuclear leukocytes, monocytes, and macrophages produce superoxide which can be converted to hydrogen peroxide by the action of superoxide dismutase. Furthermore, hydrogen peroxide, via a series of reactions known as the Haber-Weiss and Fenton reactions, can be broken down to hydroxyl radical ($OH^{\bullet}$) in the presence of iron and copper ions. Hydroxyl radicals are regarded as the most reactive of ROS and can cause damage to proteins, lipids, carbohydrates and DNA (Birden, et al, 2012).

Furthermore, oxidative stress can lead to increased production of radical generating enzymes such as xanthine oxidase, lipogenase and cyclooxygenase. The radical generating enzyme, xanthine oxidase is known to mediate the production of superoxide anion in the mitochondria, and hydrogen peroxide (Birden, et al., 2012).

The main targets of oxidative stress are proteins and DNA structure, causing DNA strand breaks, crosslinks, or sister chromatid exchanges. This can result in oxidative damage and modification of DNA, and hence a chance of mutagenesis (Niu, et al, 2015). Furthermore, reactive species affect DNA methylation by oxidizing key enzymes involved in the methylation

process (Jena, 2012). They are also known to easily initiate lipid *oxidation in vitro*, leading to the accumulation of lipid peroxidation products such as hydroperoxides and malondialdehyde (MDA), characteristic components of the first and second stages of lipid oxidation reactions, respectively (Jena, 2012). Excessive production of reactive species is known to be a possible cause of irreversible cell damage resulting in cell death by necrotic and apoptotic processes (Durackova, 2010). This has been reported as subsequently leading to adverse health conditions such as cancer, cardiovascular diseases and Alzheimer's disease among others (Shahidi and Naczk, 2004).

Living organisms have developed complex antioxidant systems to counteract reactive species and to reduce damages caused by them. These include ascorbic acid, tocopherol, uric acid, and enzymes, glutathione oxidase, glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase. They are regarded as cellular antioxidants, and together, the endogenous and dietary derived antioxidants constitute our antioxidant defence system.

Antioxidants are defined as compounds/substances that significantly reduce the adverse effects of reactive oxygen and nitrogen species. Antioxidant phytochemicals of herbs and beverage origin such as phenolic compounds (phenolic acids and flavonoids), phytic acid and selenium are known to constitute the major dietary antioxidants with possible health benefits.

The association of oxidative stress with a wide range of chronic diseases has led to the challenge to understand the role of specific antioxidants in different pathological and physiological conditions. Several animal models have been used to investigate a useful biomarker that will reflect the initiation of oxidative stress so that the quality of the antioxidant can be estimated.

Depending on reaction mechanism, antioxidants can be grouped as radical chain-breaking and preventative antioxidants. Chain-breaking antioxidants convert reactive species to stable/un-reactive compounds through a single electron transfer or hydrogen atom transfer hence leading to termination of oxidation reaction. On the other hand, preventative antioxidants inhibit the oxidation reaction from occurring by either converting the precursors of ROS to un-reactive species or halting oxidation reaction (Halliwell and Gutteridge 2007).

In the case of phenolic antioxidants such as caffeic and chlorogenic acids, and flavonoids, their health benefits are as a result of interacting with ROS/RNS by donating hydrogen atoms to reduce free radicals and to inhibit oxidation and terminate chain reactions before cell viability

is seriously affected (Kumar and Pandey, 2013). Furthermore, despite using different methodologies, there is strong evidence that chlorogenic acids are effective antioxidants that will protect against oxidation reactions in vivo by up-regulating redox-related nuclear transcription factors involved in expression of antioxidant enzymes (Hwang, et al, 2014, Shan, et al, 2009).

1.7.1.1 Anti-inflammatory effects of antioxidants

Inflammation is a natural defence mechanism against pathogens/tissue injuries caused by both exogenous and endogenous sources. The exogenous inducers/sources include microbial and viral infections, exposure to allergens, radiation and toxic chemicals, consumption of alcohol, tobacco use, and a high-calorie diet (Medzhitov, 2008), while endogenous sources/inducers of inflammation arise from autoimmune and chronic diseases due to cell signalling in response to damaged or malfunctioning tissues (Nathan, 2006).

Various inflammatory stimuli such as excessive ROS/RNS produced in the process of oxidative metabolism and some natural or artificial chemicals have been reported to initiate the inflammatory process through the nuclear factor kappa B (NF-κB) pathway. The NF-κB leads to the release of pro-inflammatory cytokines, chemokines, and adhesion molecules, which have been found to play critical role in inflammatory processes resulting in several chronic diseases (Lawrence, 2009).

Several anti-inflammatory drugs are developed to resolve conditions of abnormal inflammation by targeting inflammatory mediators or modulating the activity of cell signalling cascades involved in responding to an inflammatory signal. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used drugs for the treatment of inflammatory diseases (Laine, 2001). The cyclooxygenase (COX) pathway is the major target for NSAIDs because COX catalyzes fatty acid oxygenation to produce eicosanoids, which are the cardinal signs of inflammation. Side effects of NSAIDs include a predisposition to ulcers and bleeding in the stomach and intestines (Liang and Kitts, 2015). Thus, there is increased interest in searching for novel agents that may have anti-inflammatory activity, without inducing adverse side effects.

Phytochemicals such as polyphenols have been reported to be able to modulate the inflammatory processes (Kim, et al, 2009). Anti-inflammatory activities of the polyphenols such as quercetin, rutin, morin, hesperetin, and hesperidin have been reported in acute and chronic inflammation in animal models. Rutin is only effective in the chronic inflammatory processes especially in arthritis; and flavanones are also effective in neurogenic inflammation induced by xylene (Rotelli, et al, 2003).

1.7.2. PREVENTION AND MANAGEMENT OF POSTPRANDIAL BLOOD GLUCOSE

Excess calorie intake and reduced physical activity induces insulin resistance. Impaired glucose tolerance results from the loss of the ability of the β-cells to compensate for the insulin resistance which results in diabetes (Ceriello and Motz, 2004). The origin of type II insulin-independent diabetes mellitus and correlating morbidities such as cardiovascular diseases, hypertension obesity and hyperlipidemia have been linked to hyperglycemia, a condition characterized by an abnormal postprandial increase of blood sugar (Haffner, 1998; Dicarli, *et al*., 2003; Sowers, *et al*. 2001).

It has been reported that about 250 million of the world's population are living with these diseases (Hussain, *et al*., 2007). Furthermore, in 2007, type II diabetes and its correlated morbidities accounts for an estimated $174 billion in the US (Dall, *et al*., 2008) and about 9% of National Health Service (NHS) expenditure in the UK with the majority of costs associated with hospitalization for diabetic complications (Diabetes UK, 2008). Hence, in the UK, diabetes and its micro and macro complications have been termed as the costliest to manage (Williams, *et al*. 2001; Daniel and Andrew 2011). Due to the impact of diabetes and its correlating complications on health and its negative effect on the economy, its management has been top priority in present society.

Increase in plasma sugar is brought about by the assimilation of monosaccharide such as glucose and fructose by enterocytes of the small intestine. These monosaccharides are only made available by the hydrolysis of dietary polysaccharide by a group of hydrolytic enzymes called α-glucosidases which includes sucrase, maltase, glucoamylase, dextrinase and the pancreatic α-amylase (Elsenhans and Caspary, 1987; Bischoff, 1994; Harris and Zimmer, 1992). These α-glucosidases are found in the brush borders of the small intestine and cleave the glycosidic bonds in complex carbohydrate to release absorbable monosaccharides.

Under diabetic conditions, untreated chronic hyperglycemia enhances the production of mitochondrial and non-mitochondrial ROS, a phenomenum which leads to hyperglycemia-

induced oxidative damage. This is caused due to increase activation rate of protein kinase C (PKC) isoforms, hexosamine pathway flux, polyol pathway flux, and advanced glycation end products (AGE) (Moussa, 2008).

It has been established that the inhibition of these enzymes which aid in the breakdown of carbohydrates can significantly decrease the postprandial increase of blood glucose level after a mixed carbohydrate diet and therefore can be an important strategy in the management of type-II diabetes (Puls, *et al*, 1977).

Most epidemiological studies have established that there are many therapeutic drugs which can been extracted from microorganisms and used to treat type II diabetes. These include acarbose (Schmidt, *et al*, 1977; Jain and Saraf, 2010), Trestatin (Watanabe, *et al*, 1984), amylostatin (Murao, *et al.,* 1977), valiolamine (Horri, *et al*. 1987), miglitol and voglibose (Jain and Saraf, 2010).

However, these drugs have been known to have side effects - acarbose is linked with abnorminal disorder which includes flatulence, meteorism and possibly diarrhea for example (Bischoff *et al*., 1985; Puls *et al*., 1977). These side effects have been linked to excessive inhibition of pancreatic α-amylase, leading to the fermentation of undigested carbohydrates by abdominal bacteria (Bischoff *et al.* 1985; Horri *et al*., 1987). As a consequence, some researchers have focused on the use of herbs as alternative therapeutic drugs for the management of type II diabetes (Jaiswal *et al*., 2012; Kumar *et al*., 2011; Kwon *et al*., 2006; Prinya Wongsa *et al*., 2012).

1.7.2.1. Alpha amylase inhibition

Alpha-amylase is an endo- acting enzyme found around the digestive organs which specifically catalyses the hydrolysis the 1- 4-α-D glucosidic linkages of starch, amylos, amylopectine, glycogen and several maltodextrines to maltose and finally to glucose (Kotowaro *et al*, 2006).

Due to the role of α-amylase in the breakdown of carbohydrates, absorption of glucose with subsequent increase in postprandial blood glucose leading to type II diabetes, the inhibition of α-amylase has been considered necessary. The inhibition of α-amylase has been made possible by the use of substances referred to as α-amylase inhibitors.

Alpha amylase inhibitors (AI's) are substances that are known to inhibit the enzyme α-amylase. AI's have been classified into proteinaceaous and non-proteinacaeous inhibitors. The proteinaceaous AI inhibitors are found in cereals and legumes while the non-proteinaceous include organic compounds like acarbose, hibiscious acid, tannins, flavonoids and glucopyranosylidene-spiro-thiohydantoin (Kwon, *et. al*, 2009).

The most commonly used AI inhibitor is acarbose. Acarbose is a natural product of a bacterium (*Actinoplanes spp)*, known to have competitive inhibition over several enzymes including α-amylase and α-glucosidase. The mechanism of inhibition of these enzymes have been postulated to be as a result of its cyclohexane and nitrogen linkages which imitates the transition state for the enzymatic cleavage of glycosidic linkages (Yoon and Robyt, 2003). Franco *et al* (2007) also postulated that the inhibition activity may be due to their cyclic structures which resemble substrates at the catalytic site of α-amylase.

Phytochemicals, such as polyphenolics of herb origin have also been reported to have weak α-amylase inhibition ability *in vivo* (Mai *et al*., 2007) but inhibition of other enzymes is strong.

1.7.2.2. Alpha glucosidase inhibition

Alpha glucosidase is an enzyme that catalyses the hydrolysis of carbohydrates to glucose, which is easily absorbed into the body. In disease conditions like the non insulin dependent diabetes or type II diabetes, excess increase in postprandial blood glucose can lead to further health problems. Hence the control or inhibition of α-glucosidase will help to delay the absorption of glucose after meal.

There are few α-glucosidase inhibitors that have been used as therapeutic medications to control type II diabetes mellitus. These include acarbose, mligitol and voglibose. The mechanism of action of these inhibitors is similar but the difference is that acarbose is an oligosaccharide while mligitol resembles a monosaccharide. Mligitol is said to be easily absorbed by the body while acrabose does not. Acarbose inhibits both pancreatic α-amylase and α-glucosidase. Considering the side effects of these inhibitors, safer inhibitors without side effects are being researched.

Just like the α-amylase, several researchers have reported the α-glucosidase inhibition ability by several herb photochemical (Jaiswal *et al*., 2012; Kumar *et al*., 2011; Kwon *et al*., 2006; Prinya Wongsa *et al*., 2012), however all reported work was undertaken on dried herbs. No

work has been carried out comparing the effects of freezing on enzyme inhibition, nor have there been any reports on fresh herbs.

1.7.3. PREVENTION AND MANAGEMENT OF HYPERTENSION / INHIBITION OF ANGIOTENSIN I-CONVERTING ENZYME

One of the deadliest complications of long-time type 2 diabetes is hypertension or high blood pressure. Persistent high blood pressure is one of the risk factors of cardiovascular diseases and chronic renal failure (Stevens, et al., 2016).

The rennin and pulmonary angiotensin I- converting enzyme (ACE), an exopeptidase enzyme is an important circulating enzyme which is involved in maintaining arterial vascular tension and mediating extracellular volume (salt and water balance) (Hernandez & Harrington, 2008). It is a main component in the renin angiotensin aldosterone system (RAAS) which regulates blood pressure.

ACE catalyzes the conversion of the histidyl-leucine dipeptide angiotensin I, into a potent octapeptide vasoconstrictor called angiotensin II. (Zhang *et al*. 2000). It also functions to degrades bradykinin, a potent vasodilator and other vasoactive peptides (Imig, 2004). When produced, angiotensin II also stimulates the synthesis and release of aldosterone, which increase blood pressure by promoting sodium retention in the distal tubules (Tingting *et al*., 2011). These actions make ACE inhibition a therapeutic approach in the treatment of some cardiovascular conditions such as high blood pressure, heart failure, diabetic nephropathy and type 2 diabetes (Kwon *et al*., 2006; Tingting *et al*., 2011).

Several potent synthetic inhibitors of ACE such as captopril and enalpril have been discovered and used in the control of hypertention, congestive heart failure and chronic renal disease (Behnia *et al*, 2003). However, these have been associated with some noticeable side effects on health such as allergic reactions, skin rashes and taste disturbances (Jimsheena and Gowda (2009). Hence many research groups are searching for novel ACE inhibitors from food components and other natural sources with minimal or no side effects.

Angiotensin I-converting enzyme (ACE) is a membrane-bound glycoprotein located in the epithelial cells of the pulmonary capillaries (Vermeirssen, *et al*., 2002; Jimsheena and Gowda 2009). ACE is associated with the blood pressure regulation system of rennin-angiotensin and can bring about the increase of blood pressure by converting decapeptide angotensin I into potent vaso-constricting octapeptide angiotensin II (Wan *et al*, 2013). Hence, ACE has been

recognised as critical in the rennin-angiotensin-aldosteron system and its connection to hypertension (Vermeirssen *et al*., 2002).

The synthetic ACE inhibitors captopril, enalpril, ramipril and lisinopril which are developed based on the venom of the Brazilian viper (*Bothrops jararaca*) are drugs used to treat mild to moderate hypertension. However, although remarkably effective, these synthetic inhibitors are associated with certain side effects. Hence, it is now believed that screening herb extracts for inhibition of ACE will be an effective method to search for new anti-hypertensive agents and sources (Kwon *et. al*, 2006; Tingting *et. al*, 2011).

Furthermore, a few antihypertensive drugs which are said to be high in dietary polyphenolics have been isolated from a number of plant species (Actis-Goretta *et al.* 2003, Kang *et al.* 2003).

1.7.4. CONTROL OF CARDIOVASCULAR DISEASES

The major risk factors of cardiovascular diseases have been recognized over many years to include high levels of low-density lipoprotein (LDL) cholesterol, smoking, hypertension, diabetes, abdominal obesity, psychosocial factors, insufficient consumption of fruits and vegetables, excess consumption of alcohol, and lack of regular physical activity (Fuster, 2014).

Apart from the treatment of cardiovascular risk factors with pharmacological agents and the use of antithrombotic drugs, there is growing awareness of the role of dietary factors and herbal medicines in the prevention of cardiovascular disease and the possibility of their use in treatment (Liu. X., 2013) .

There are few clinical studies on the effects of consumption of herbs on the management of cardiovascular diseases in humans, although Costa et al have reported the ability of lemon grass oil to lower cholesterol in mice (Costa, 2011).

1.7.5. ANTI-CANCER THERAPY

One of the anti-cancer mechanisms of herbs has been reported as to be through increase in endogenous protective enzymes, protecting DNA from free radical-induced structural damage and encouraging self-destruction of abnormal cells, hence inhibiting the growth of tumours (Hedges and Lister, 2007).

It is estimated that approximately two thirds of cancers can be prevented by appropriate changes in diet and lifestyle and people who eat more than five servings of fruits and vegetables

per day reduce their chances of developing cancer by 50% compared to people who eat less than 2 servings (Surh, 2003). Some of the chemopreventive phenolic compounds from common foods sources are catechins from tea (Yoo and Chul-Ho, 2014), curcumin from turmeric (Vaishali, 2013), and gingerol from ginger (Wang, *et al*, 2015).

Irrespective of the chemopreventive effects of individual phenolic compounds, it has been suggested that better beneficial effects can be obtained from the synergistic effects of more than one phenolic compound.

1.8 AIMS AND OBJECTIVES

It is generally accepted that there are dietary and health benefits associated with a range of foodstuffs including herbs of the *lamiceae* family. However, most food composition tables which are necessary tools for epidemiological and nutritional studies only represent foodstuffs consumed in their raw state. These do not consider the fact that bioavailability and activity of some constituents may be affected by certain home storage and processing methods such as blanching and freezing (Canet, *et al*, 2004; Chan, *et al*, 2014; Jaiswal, *et al*, 2012). Most commonly discussed is the instability and thermal degradation of ascorbic acid due to blanching, cooking and dehydration. Hence, there is no clear evidence as to the best way to store and prepare these to achieve maximal health benefits.

To investigate this, I will examine the effects of freezing and blanching compared to fresh herbs for 3 members of the *lamiceae* family across a range of functional properties and constituents reported to be related to good health.

AIMS

The aim of this work is to test 3 common members of the *lamiceae* family of herbs – mint/*Mentha piperata*, basil/*Ocmium bascilla* and thyme/*Thymus vulgaris*. I will investigate the anti-oxidant properties of each as well as the level of ascorbic acid, selenium, phytic acid and total phenolic content as functionally important constituents associated with health benefits.

OBJECTIVES

To achieve my aims, I will use a range of methodologies including DPPH, FRAP, ORAC and CUPRAC to assess antioxidant properties under a range of herb treatments. Functional constituents will be measured by a range of biochemical techniques including HPLC, spectrophotometric, atomic absorption and spectrofluorometric analysis. My overall objective will be to identify whether there are differences between frozen, blanched and fresh herbs of the *lamiceae* family in terms of functional properties and constituents relevant to health.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 PLANT MATERIALS

Fresh herbs in pots from Lincolnshire herbs were purchased from a local supermarket in Lincoln, UK. All herbs were randomly purchased on different dates for repeat experiments.

2.1.2 CHEMICALS AND REAGENTS

Phenolic acid standards (Protocatechuic, p-hydroxybenzoic acid, chlorogenic acid, caffeic acid, catechin, ellagic acid, ferrulic acid, rosemarinic acid, p-coumeric acid) HPLC grades (99-100%) were purchased from Sigma –Aldrich Ltd, Fancy road Dorset. Gallic acid was purchased from Acros organics (New Jersey, USA).

Reagents for ascorbic acid assays, dithiothreitol (DTT), N-ethylmaleimide (NEM) and α,α-bipyridyl were all purchased from Sigma Aldrich UK. Trichloroacetic acid and orthophosphoric acid were obtained from Fisher, scientific, UK.

HPLC grade mobile phase (methanol, acetonitrile) were all obtained from Fisher scientific chemicals, Bishops meadow road, Loughborough, UK

Antioxidant reagents, methylated β-cyclodextrin, disodium fluorescien, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), ascorbic acid, 2, 2′-azobis amidnopropane di-hydrchloride (AAPH), 2,2′-diphenylpicrylhydrazyl reagent, 4, 6-tripyridyl-s-triazine (TPTZ), Sodium bicarbonate and Folin-ciocalteu phenol reagent were purchased from Sigma-Aldrich, UK. Fe III chloride and copper II chloride were obtained from Fluka Sigma, UK.

Reagents for buffer solutions, mono and dibasic potassium and sodium phosphate were all obtained from Sigma-Aldrich, UK

Reagents for enzyme inhibition assay, enzymes porcine pancreatic α-amylase and *saccharomyces cereviceae* α-glucosidase, substrates p-nitrophenyl glucosidase (pNPG) and potato starch, 3,5-dinitrosalicyclic acid (DNSA) were all obtained from Sigma-Aldrich, UK.

Reagents for trace mineral analysis, for phytic acid assay; phytic acid from rice and DOWEX anion exchange resin and sodium selenite and selenate for selenium analysis were all obtained

from Sigma-Aldrich, UK. Selenium standard, matrix modifiers (Magnesium nitrite) were purchased from Fisher scientific, UK.

For the probiotic bacterial viability assay, all pure cultures of *lactobacillus rhamnosus* and *bifidobacteria bifidum* were purchased from the culture collection centre of the Ministry of health, UK. Culture media were prepared using Mann-ragosa Sharpe agar purchased from Oxoid, UK.

Ultra pure deionised water was purified by Millipore ultra pure system was used throughout the work

2.1.3 EQUIPMENTENT/INSTRUMENTS

Spectrophotometric absorbance readings were carried out using a Shimadzu UV-1601 spectrophotometer (Shimadzu Corporation, Tokyo Japan) with 1cm plastic disposable Startz cuvettes.

ORAC fluorometric assay and α-glucosidase enzyme inhibition assays were determined using the BMJ services plate reader with blank plastic disposable 96-well plates.

HPLC apparatus La Chrome, Merck, Hitachi, Ltd (Tokyo Japan) equipped with an auto sampler Hitachi L-7200, Ltd, (Tokyo, Japan) and a diode array detector (DAD, Hitachi L-7455, Ltd (Tokyo, Japan).

Perkin-Elmer AAnalyst 800 atomic absorption spectrometer (Norwalk CT, USA) equipped with HGA graphite furnance and deuterium background corrector.

Avanti J-25 centrifuge with JA-2550 fixed angle rotor from Beckman Coulter, California, USA

Electric furnance, Carbolite, Bamford, Sheffield, England.

Analytical balance (max 220g) BP 221S Sartorius, Germany

Analytical balance (max. 510g) AR5120 Adventurer, Ohaus, China

2.2 METHODS

2.2.1 PREPARATION OF PLANT MATERIAL

The blanching time and temperature were optimized by ascorbic acid and phytic assay of mint/*Mentha piperita* at different conditions (blanching at temperatures 100˚C, 80˚C and 50˚C for times 3, 5 and 10mins). The best blanching time and temperature (100˚C for 3mins) which showed the least ascorbic acid loss was then employed for the rest of the assays.

From fresh pots, 3 batches of known weight (10-20g) were prepared. One batch was extracted while fresh, a second batch wrapped in polyethylene freezer bags and stored in a freezer compartment (-20˚C) the last batch is water blanched (immersion), at 100˚C for 3 minutes, drained and then sprinkled with cold water from the tap to cool. After being allowed to drain, these were wrapped in polyethylene freezer bags and put in the freezer compartment (-20˚C).

2.2.2 MOISTURE CONTENT DETERMINATION

Moisture content of herbs was determined by the AOAC official method for plants by oven drying (AOAC 1995, No. 934.06). A clean empty metal dish containing about 1g of sand was dried in the oven for approximately 3 hours and placed into a dessicator to cool down, then weighed. Samples were macerated using mortar and pestle. Approximately 1g of sample was accurately weighed (± 0.05g) into the metal dish with sand and with added water (1ml) homogenised with a glass rod and dried in the oven at 60˚C for 24hrs. The samples were then cooled in a dessicator and reweighed. Moisture content was determined and calculated as

Moisture content = weight of wet sample – weight of dry sample

$$\% \text{ Moisture content} = \frac{\text{weight of wet sample} - \text{weight of dry sample}}{weight\ \ of\ wet\ sample} \times 100$$

2.2.3 TOTAL ANTIOXIDANT CAPACITY ASSAYS

Total antioxidant capacity of plant foods practically deals with the synergistic action of a wide variety of antioxidants such as vitamins C and E and polyphenols, carotenoids, terpenoids, Millard compounds and trace minerals (Ou *et al*., 2002).

There has been growing interest in the measurement of the antioxidant capacity of food products. This has led to good information about food systems, such as resistance to oxidation, quantitative contribution of antioxidant substances, or the antioxidant activity provided by the food when ingested (Huang *et al*., 2005.; Serrano *et al*., 2007).

There are many *in-vitro* methods for analysis of antioxidants in food stuffs. These tests used are mostly indirect methods which measure the ability of antioxidants in a material to inhibit the oxidative effects of reactive oxygen species which are resolutely generated in the reaction mixture.

Among the many methods for the in vitro analysis of potential antioxidants in foodstuffs, there are those that measure the ability of the antioxidant to break the chain reaction of lipid peroxidation (Schleiser, Harwat, Bohm, and Bitsch, 2002; Roginsky and Lissi, 2005), those that absorb and neutralize free radicals (Osawa, 1999) and those that bind metal ions with the formation of a complex (Yoshida *et al*., 2003) In this thesis, three electron transfer antioxidant assays (FRAP, DPPH and CUPRAC) and a hydrogen atom transfer antioxidant assay (ORAC) were utilised.

2.2.3.1 OXYGEN RADICAL ABSORBTION CAPACITY (ORAC)

The ORAC assay is based upon an early works of Glazer (1990) and Ghiselli *et al*., (1995) but was further developed by Cao *et al*., (1993) The ORAC assay is a method to determine the scavenging capacity of compounds with antioxidant activity against free radicals that are produced from the azo-radical initiator AAPH (2,2-azobis(2-amidinopropane) dihydrochloride) (Prior, *et al*., 2003). The assay measures the free radical oxidation of a fluorescent probe through the change in its fluorescent intensity (Cao and Prior, 1999).

The principle of the improved ORAC assay shows the effect of peroxyl radicals (ROO) generated from the thermal decomposition of 2, 2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) on the signal intensity from the fluorescent probe, fluorescein, in the presence of an oxygen radical absorbing substance. The stronger the absorbing capacity, the more the peroxyl radicals are quenched, thus maintaining the intensity of the fluorescent signal observed using fluorometers. In order to obtain a result, the area under the curve of the fluorescence intensity versus time is subtracted from that of a negative control sample to determine the antioxidant capacity of the substance present, in trolox equivalents (TE).

2.2.3.1.1 ORAC OF HERB MATERIALS

In this research, ORAC was performed as described by Gillespie *et al.* (2007) with slight modification. For this analysis, 0.08µM fluorescien was used as the target compound using and 150mM AAPH prepared in 75mM phosphate buffer. Trolox concentrations of 12.5-100 µM dissolved in extraction solvents and made up with phosphate buffer were used as standard and 40µM was used as a control. The assay was performed using a microplate and is assessed with a 96-well multi-detection plate reader running a fluorescence kinetic read with excitation wavelength of 485nm and an emission wavelength of 520nm. The assay temperature was 37˚C and had a duration of 1hr.

2.2.3.1.2 ASSAY METHOD

150µl 0.08 µM fluorescin was added in each well of a black plate. 25 µl phosphate buffer (blank), trolox standard or sample and 100 µl phosphate buffer was then added to each well. The mix was incubated for 15mins at 37˚C. 25µl AAPH was added to each well, the plate placed in the plate reader shaken for 10 seconds and fluorescent intensity taken every 5 mins until 90% of fluorescent intensity has declined. The final ORAC values were calculated using a regression equation between the Trolox concentration and the net area under the fluorescin decay curve and expressed as Trolox equivalents as micromoles per 100gram dry weight. The area under the decay curve (AUC) was calculated as;

$$AUC = 0.5 + (f_5/f_0 + f_{10}/f_0 + f_{15}/f_0 + f_{20}/f_0 + \ldots\ldots + f_{55}/f_0 + f_{60}/f_0) \times 5$$

Where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*. Net AUC was calculated by subtracting the AUC of blank from sample and standard.

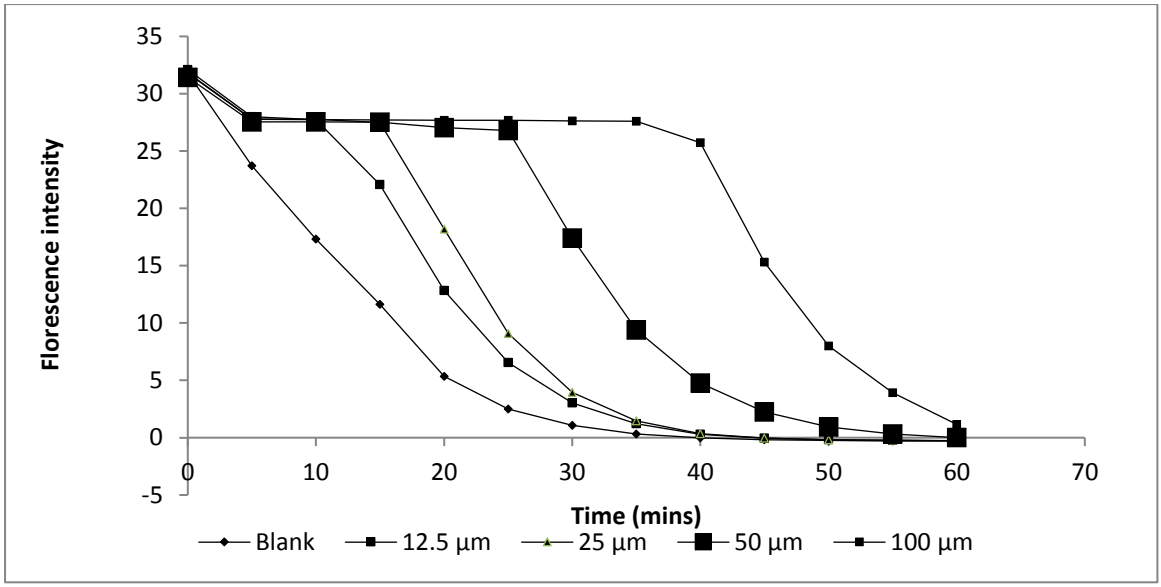


Figure 2.1 ORAC fluorecein decay curve in the presence of different Trolox concentrations

2.2.3.2 FERROUS REDUCING ANTIOXIDANT POTENTIAL (FRAP)

FRAP is known as the ferric reducing potential. FRAP as originally developed by Benzie and Strain (1996) was made to measure reducing power in plasma, but recently, the assay has also been adapted and used to assay antioxidants in botanicals (Benzie, 1996. Benzie and Szeto, 1999; Ou, *et al*, 2002; Gil, 2000; Pellegrini, *et al*., 2003; Protggente, *et al*., 2002)

It is a reaction that happens at low pH (3.4) and is characterized by the reduction of ferric tripyridyltriazine (Fe^{III}-TPTZ) complex to ferrous (Fe^{II}) form (Fe-$TPTZ^{2+}$) with a resultant formation of an intense blue colour with an absorption maximum at 593 nm. It is commonly referred to as the Fe^{3+} (an active propagator of radical chains) to Fe^{2+}reducing activity. It is an electron transfer mechanism and so in combination with other assay methods may be useful to distinguish dominant mechanisms with different antioxidants (Prior, *et al.*, 2005).

However, as has been suggested by some researchers (Wong, Li, Cheng, and Chen 2006; Katalinic, *et al*., 2006) the reducing capacity does not necessarily reflect total antioxidant activity and does not measure thiol group antioxidants such as gluthatione but reflects the antioxidants that reduce Fe ion.

2.2.3.2.2 ASSAY METHOD

The FRAP assay was carried out according to Benzi and Strain (1996) as modified by Thaipong *et al*. (2006). Fresh working FRAP stock solution was made up of acetate buffer, 2, 4, 6-tripyridyl-s-triazine (TPTZ) and iron (III) chloride solution. FRAP working solution is warmed at 37˚C before use.

Preparation of standards and controls

Aqueous solutions of known concentration of Fe (II) ($FeSO_4$:$7H_2$ O) and a freshly prepared pure antioxidant (trolox) were used for the calibration of the FRAP assay. Fe (II) as used is said to represent a one electron exchange reaction and is taken as unity, that is, the blank corrected signal given by 100µM solution of Fe (II) is equivalent to a FRAP value of 100µM (Benzie, 1999).

For this research, a straight-line curve was obtained by mixing 150µl of trolox standard solutions of concentration ranging from 100-1000µM and 2.850ml of the working solution. Trolox is said to have a stoichiometric factor of 2.0 in the FRAP assay, hence, the direct reaction of Fe (II) gives a change in absorbance half that of an equivalent molar concentration of trolox (Benzie and Strain, 1996). Hence a trolox concentration of 500µM is equivalent to 1000µM of antioxidant power as FRAP.

Fresh mixtures of plant extracts and working FRAP mixture were covered with aluminium foil and allowed to stand in the dark at room temperature for 30 minutes. The change in absorbance was then recorded at 593nm. In addition, known concentrations of pure antioxidant standards like catechin, ascorbic acid and quercetin were used as controls to monitor accuracy and precision.

The mixtures were prepared fresh on a daily basis with a parallel assay of known standards and the test materials. This is to monitor accuracy of the test and ensure comparable results.

2.2.3.3 CUPRIC REDUCING ANTIOXIDANT CAPACITY (CUPRAC)

The CUPRAC method is evolving into an antioxidant measurement assay in food chemistry and biochemistry and has shown distinct advantages over some antioxidant assays (Ozyurek, *et al*., 2011). The CUPRAC method uses a cupric neocuproine (2,9-dimethyl- 1,10-phenanthroline) chelate – abbreviated as (Cu(II)-Nc) – as the chromogenic oxidant and is based

on the redox reaction with antioxidants producing the cuprous-neocuproine chelate – abbreviated as (Cu(I)-Nc) – showing maximum light absorption at 450 nm (Apak, *et al*., 2004). The reaction equation with n-electron reductant antioxidants can be formulated by:

$nCu(Nc)_2^{2+}$ + n-e reductant ⎯→ $nCu(Nc)_2^{+}$ + n-e oxidized product + nH

In a recent paper review (Apak, *et al*., 2007), elucidated numerous advantages that CUPRAC has over other antioxidant assay methods. These include CUPRAC's ability to respond much faster than FRAP to certain hydrocinnamic acids. This is attributed to their respective electronic configurations involving the kinetic inertness of high-spin of half-filled d-orbitals of Fe^{3+} (d^5) of FRAP and the faster kinetics of CUPRAC's Cu^{2+} filled d-orbital (d^9). Furthermore, it is carried out using a buffer (ammonium acetate) at a more realistic physiological pH 7, quite unlike a more impractical acidic pH of the FRAP which may adversely affect antioxidant analysis. This is because extreme acidic condition can lead to protonation of phenolics, therefore resulting in the suppression of the reducing capacity of antioxidants. Additionally, CUPRAC is said to simultaneously measure hydrophilic as well as lipophilic antioxidants (e.g., β-carotene and α-tocopherol) and include the thiol (GSH) group.

2.2.3.3.1 CUPRAC OF PLANT MATERIALS

CUPRAC was performed as described by Apak *et al*., (2004) using $CuCl_2$ solution, ammonium acetate buffer ph 7.0 and neocuproine (Nc) solution.

A straight-line curve was obtained by mixing 150µl of trolox standard solution of concentrations ranging from 100-500µM and 1ml of copper (ii) chloride, 1ml of ammonium acetate buffer, 1ml of neocuproine solution and 750 µl of distilled water. The mixture was incubated at 50◦C for 20mins and the absorbance read at 450nm.

2.2.3.3.2 SAMPLE ASSAY METHOD

150µl of sample extract properly diluted with methanol (where necessary) was allowed to react with 1ml of copper (II) chloride, 1ml of ammonium acetate buffer, 1ml of neocuproine solution and 750 µl of distilled water. The mixture was incubated at 50◦C for 20mins. The absorbance was taken at 450nm. Results are expressed in Mg TE/g dw of plant material.

The mixtures were prepared fresh on a daily basis with parallel assay of standards and the test materials. This was to monitor accuracy of the test and ensure comparable results.

2.2.3.4 DIPHENYL PICRYL HYDRAZYL SCAVENGING ASSAY (DPPH)

This spectrophotometric assay uses the stable 2,2′-diphenylpicrylhydrazyl (DPPH) radical as a reagent, which loses its absorption when reduced by an antioxidant or free radical species (Burits and Bucar 2000; Cuendet, *et al*., 1997) and therefore has been widely used to determine antiradical/antioxidant activity of purified phenolic compounds as well as natural plant extracts (Brand-Williams, *et al*. 1995; Sripriya, *et al*. 1996; Bondet, *et al.*, 1997; Mahinda and Shahidi 2000; Peyrat-Mailard, *et al*., 2000; Fukumoto and Mazza 2000).

DPPH is a stable nitrogen free radical that shows a maximum absorption at 517 nm in methanol. When DPPH encounters proton donating substances such as an antioxidant and any radical species, the absorbance at 517 nm disappears with a pale-yellow colour from the pycryl group because the DPPH radical is scavenged (Marinoya and Yanishlieva, 1997). It is the measurement of the ability of an antioxidant to reduce DPPH. The ability is either evaluated by electron spin resonance (ER) or by measuring the disappearance of its absorbance (Prior, *et al*., 2005)

DPPH colour can be lost via either radical reaction (HAT) or reduction (SET) as well as unrelated reactions, and steric accessibility is a major determinant of the reaction (Prior, *et al.*, 2005). Furthermore, due to steric inaccessibility, many antioxidants that react quickly with peroxyl radicals may react slowly or even be inert with DPPH.

The hydrogen atoms or electron-donation ability of the corresponding extracts of some pure compounds were measured from the bleaching of a purple-coloured methanol solution of DPPH. Guo, *et al*., (2001) reported that DPPH works very well in ethanol and methanol which do not interfere with the reaction compared to other solvent systems such as acetone or water, which seem to give low values for the extent of reduction.

Other drawbacks associated with DPPH assay is the fact that it is not a competitive reaction because DPPH is both a radical probe and an oxidant; it is decolourized by hydrogen atom transfer and reducing agents leading to inaccurate antioxidant capacity calculation (Prior, *et al*., 2005).

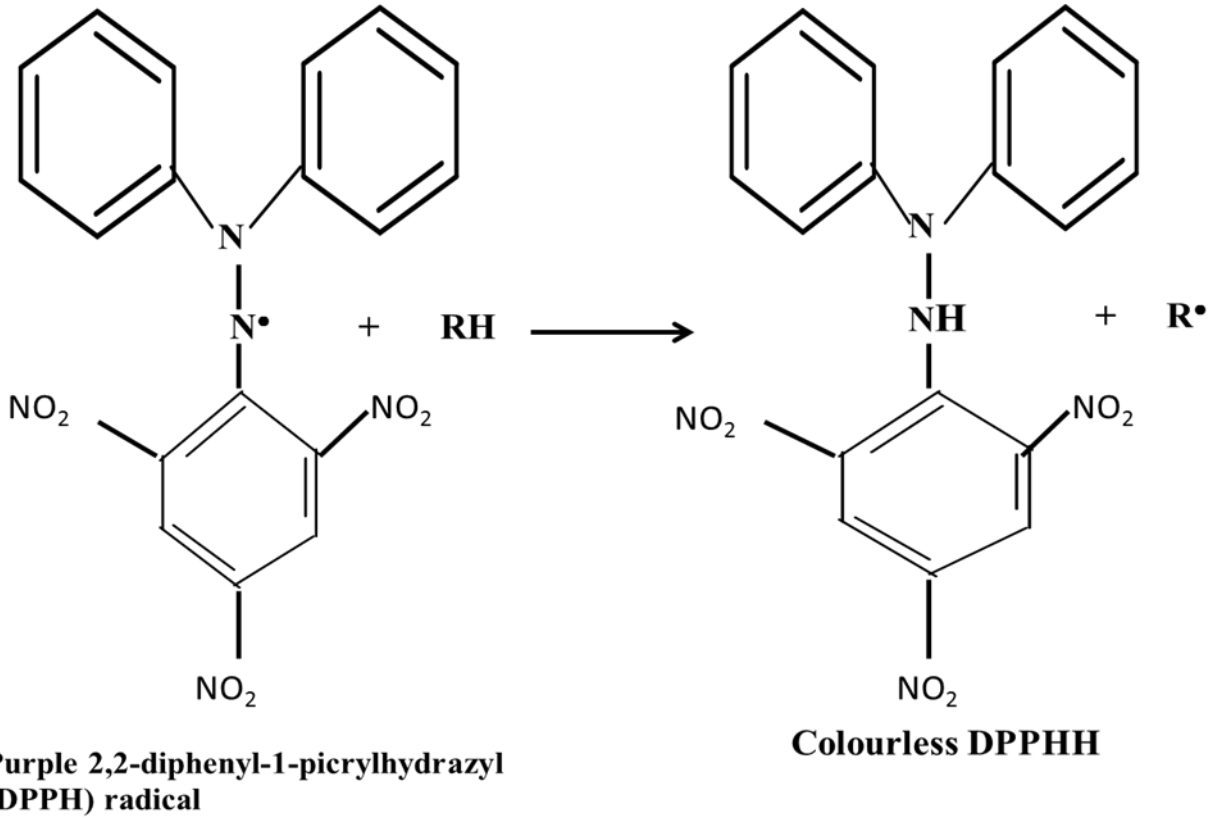


Figure 2.2 Representation of DPPH scavenging activity by antioxidant (RH)

2.2.3.4.1 DIPHENYL PICRYL HYDRAZYL SCAVENGING ASSAY (DPPH)

The DPPH assay was carried out according to Brand –Williams *et al* (1995) with slight modification. 1mM stock solution of DPPH was prepared in 99.9% methanol and stored at -20˚C until required. A 136µM working solution of DPPH was then prepared.

A straight-line curve was obtained by mixing 150µl of standard solutions of trolox concentrations of between 100-500µM and 2.850ml of the working solution. The mixture was then covered with aluminium foil and allowed to stand in the dark at room temperature for 30 minutes. The absorbance was taken at 515nm.

The mixtures were prepared fresh on a daily basis with a parallel assay of standards and the test materials. This was to monitor accuracy of the test and ensure comparable results.

2.2.3.4.2 SAMPLE ASSAY

For DPPH analysis, 150µl of sample extract and 2.850ml of DPPH were mixed, covered with aluminium foil and incubated at room temperature for 30 minutes. Absorbance was recorded at 515nm. The IC_{50} value for each sample, defined as the concentration (in mg) of the test sample leading to 50% reduction of initial DPPH concentration, was calculated from the linear regression analysis. Antioxidant activity is expressed as mg TE/g of dw of plant material.

Percentage inhibition/radical scavenging activity (RSA) was calculated by:

$\frac{Ao-Ai}{Ao}$ x 100

Ao is the absorbance of blank/control; Ai is the absorbance of sample extract/standard. The IC_{50} was determined from the excel plot of concentration of extract and the percentage inhibition.

2.2.4 PHENOLIC COMPOUNDS/TOTAL PHENOLIC CONTENT ASSAY

A number of both spectrophotometric and chromatographical methods have been developed for the identification and quantification of phenolic compounds in materials. These assays are based on different principles and are employed for the separation and determination of various structural phenolic groups. Most of the assays involve the use of high performance liquid chromatography (HPLC) with a UV spectrophotometer.

Most researchers prefer a common determination of the total phenolic content (TPC). The most common method available for TPC of materials is the spectrophotometric method utilizing the Folin-Ciocalteu (FC) reagent as developed by Singleton and Rossi (1965). The Folin-Ciocalteu method is an indirect determination of TPC of materials through the reducing capacity of components of materials under observation. The mechanism involves the transfer of electrons in an alkaline medium from the phenolic compounds to the Folin-Ciocalteu reagents made up of phosphotungastic/phosphomolybdic complex (Singleton and Rossi, 1965). The resultant blue colour formed from the redox reaction between the reducing phenolic compounds and Folin-Ciocalteu reagent is measured spectrophotometrically at 725nm. Concentration of phenolic content of each sample is calibrated against gallic acid and recorded as gallic acid equivalent (GAE).

2.2.4.1 SAMPLE ANALYSIS

Total phenolics content was determined using the Folin-Ciocalteu method. To a 4ml cuvette, 200 µl of plant extract was mixed with 1.5ml of Folin-Ciocalteu reagent (already diluted, 10-fold with water) and allowed to stand at 22˚C for 5 minutes, after which 1.5ml sodium bicarbonate (60mg/ml) was added to the mixture. After 90 minutes at 22˚C absorbance was measured at 725nm using a spectrophotometer.

A linear calibration curve of Gallic acid in range of 100 - 500µg /ml was constructed equation $y= 0.0048x – 0.0463$ and $R^2 = 0.9973$. Total phenolic content was expressed as mg Gallic acid equivalent (GAE). Samples are measured in triplicates and mean values determined and recorded.

The mixtures were prepared fresh on daily basis with parallel assay of standards and the test materials. This is to monitor accuracy of the test and ensure comparable results.

2.2.5 HPLC ANALYSIS OF INDIVIDUAL PHENOLIC COMPOUNDS OF HERBS

Phenolic acid content of water extracts of herbs was determined as described by Kwon *et al*. (2006) with slight modifications. 2ml aqueous extract of herb was filtered through a 0.45µm filter. 5µl volume of the filtered sample extract was injected in a HPLC apparatus (La Chrome, Merck, Hitachi, Ltd, Tokyo Japan) equipped with an autosampler (Hitachi L-7200, ltd, Tokyo, Japan) and diode array detector (DAD, Hitachi L-7455, Ltd Tokyo, Japan). Solvents used for gradient elution were 10mM phosphorous acid (ph 2.6) and 100% methanol. The methanol was increased to 60% for first 8 mins and to 100% over the next 7mins and 0% for the next 3mins and was maintained for the next 10mins (total run time 28mins). Phenolic compounds; caffeic acid, ellagic acid, chlorogenic acid, protocatechuic acid, p-coumeric acid, catechin, ferrulic, hydrobenzoic acids contents were identified using a separation analytical column (Agilent C-18, 250 x4.6mm i.d) packed with material of 5µm particle size at a flow rate of 0.5ml per min at room temperature. During each run the absorbance was recorded between 240 and 333nm. Peak identification was performed by comparing the retention times and the diode array chromatograms of standard phenolic acids in 100% methanol.

Phenolic phytochemical content of herbs was determined from a calibration curve of the standards of pure phenolic acids ((purchased from Sigma Chemical Co., St. Louise, MO). The results were expressed as mg/g of dry weight of herbs.

2.2.6 ASCORBIC ACID ASSAY

The most used method in the detection of ascorbic acid in different samples has been the high-performance liquid chromatography (HPLC) with different detectors (Novakova *et al*, 2008). The HPLC offers good repeatability, accuracy, reproducibility and unambiguous identification of ascorbic acid. However, it is both time intensive and a limited number of samples can be analysed at a time.

Hence, Gillespie and Ainsworth (2007) developed a more rapid spectrophotometric method for the analysis of all forms of ascorbic acid (reduced, oxidized and total ascorbate based on the method of Okamura (1980). This method involves the reduction of ferric ion by ascorbic acid to its ferrous form product of which when coupled with α-α-bipyridyl forms a complex with characteristic absorbance at 525nm.

2.2.6.1 ASCORBIC ACID ASSAY OF SAMPLES

Analysis was carried out according to Gillespie and Ainsworth (2007) with slight modification. Approximately 160mg of plant material was homogenised with 1ml 6% TCA solution using a mortar and pestle in an ice bath. Extracts were collected in a screw cap vial. Mortar and pestle were rinsed with 3ml 6% TCA solution and collected in the same tube. Samples were centrifuged at 1300g for 5 min. Supernatants were decanted into screw capped tubes and kept in an ice bath.

2.2.6.1.1 REAGENT SET UP

- 6 and 10% (wt/vol) solutions of trichloroacetic acid (TCA) were prepared and kept at room temperature (*~20 ˚C)*.
- 10mM solution of dithiothreitol (DTT) was prepared and kept in an ice bath
- 0.5% (wt/vol) of N-ethylmaleimide (NEM) and kept in an ice bath.
- 43% (vol/vol) of phosphoric acid ($H_3\ PO_4$) solution was prepared (a 1:1 dilution of 85% reagent-grade $H_3\ PO_4$). Keep at room temperature.
- 3% (wt/vol) of $FeCl_3$ was prepared and kept at room temperature.
- 4% (wt/vol) solution of α,α-bipyridyl. Keep in an ice bath.
- 75 mM phosphate buffer (pH 7.0) was prepared from potassium phosphate, monobasic and potassium phosphate, dibasic.
- 150-750µM of ascorbate standards was prepared in 6% (w/v) TCA. Keep the ascorbate standards in an ice bath

2.2.6.1.2 PROCEDURE

EXTRACTION OF SAMPLES

Appropriately weighed cut herbs (approximately 1g) were immediately put into screw capped tubes and frozen in liquid nitrogen, then further homogenized under ice bath using a chilled mortar and pestle. Add 4ml 6% TCA and transfer to a 25 ml tube. The mortar and pestle were further washed with another 4 ml of 6% TCA and collect in the same tube. Samples were centrifuged (13,000g for 5 min at 4 ˚C). The supernatants were transferred into a further 25ml tube and kept in an ice bath prior to analysis.

ASCORBIC ACID ASSAY

Blanks, standards and samples were assayed in triplicates for both reduced AA and total AA (six assays per sample). Add 100 µl 75 mM phosphate buffer and 200 µl of either 6% TCA (blank), AA standards (150-750µM) or sample to a 4-ml tube. 100 µl 10 mM DTT was added to the total AA tubes and incubate at room temperature for 10 min. This reduced the pool of oxidized AA. 100 µl 0.5% NEM was added to the total AA tubes to remove the excess DTT and incubated for at least 30 s. 200 µl of water was added to the reduced AA assay tubes to account for the volume of DTT and NEM added to the total AA assay tubes. Finally 500 µl 10% TCA, 400 µl 43% H3PO4, 400 µl 4% α,α-bipyridyl and 200 ml 3% FeCl3 was mixed intoto all assay tubes. Assay tubes were incubated at 37 ˚C for 1 h. Absorbance was read at 525nm.

Total and reduced ascorbic acid was calculated from the standard curve equation obtained from a straight-line graph of ascorbic acid concentrations of between 150-750µM. Oxidized ascorbic acid was calculated as the difference between TAA and RAA. The mixtures were prepared fresh on daily basis with parallel assay of standards and the test materials. This was to monitor accuracy of the test and ensure comparable results.

2.2.7 DETERMINATION OF SELENIUM CONTENT

For selenium content determination, sample extraction is very important to convert the whole sample to an analysable form. Extraction efficiency and method should depend on the nature of sample and the extraction conditions. Extraction methods should be well considered to prevent losses and minimize changes in compound. For plants, common techniques employed include enzymatic hydrolysis, heating under reflux to leach out active components (Wrobel

and Caruso, 2005), microwave extraction (Guo *et al*., 2001; Mingorance, 2002). Enzyme hydrolysis assisted with ultrasounds has been reported to provided better extraction time without component degradation (Sanz-Medel *et al*., 2006). Furthermore, the use of proteolytic enzymes ensures the liberation of selenium species contained in peptides or proteins. Extraction using an aqueous solution of enzyme-deactivating hydroxylamine hydrochloride counteracts the possible degradation of selenium labile species by enzymes that occur naturally in garlic (Larsen *et al*., 2006).

Generally, 100% solubilisation efficiency has been reported for selenium species using enzymatic treatment and lower (75-90%) using hot water reflux using *allium* vegetables. However, enzymatic treatment give more of Se_{Met} (Larsen *et al*., 2006). Furthermore, extractability using water varied among plant materials. For instance, 68.5% of total selenium was obtained from selenised Japanese pungent radish (Ogra et al., 2007), but 67% in chicory leaves (Mazej *et al*., 2006). Finally, Kolachi *et al*. (2010) did not obtain a significant difference between conventional and microwave-assisted selenium extraction.

2.2.7.1 ASSAY METHODS

Several methods have been employed for the determination of selenium. These include spectrophotometric, atomic absorption spectrometry (AAS), electrochemical techniques, spectroflurometry, neutron activation analysis and chromatography. Selection of an assay method depends on the specie of selenium to characterize. Vonderheide *et al*. (2002) utilized high performance liquid chromatography (HPLC) with an inductively coupled plasma mass spectrometer to characterize selenium species selenomethionine (Se_{Met}), selenoethionen (SeE_t) and selenocystein (Se_{Cys}) in Brazil nuts and further used electrospray mass spectrometry (ES-MS) for unidentifiable selenium species. However, AAS is used mainly for general selenium determination.

2.2.7.2 SELENIUM CONTENT DETERMINATION OF SAMPLES

2g of replicate samples of plant materials (fresh, un-blanched and blanched frozen) were ground using a marble mortar and pestle. Ground samples were taken into Pyrex beakers (50 mL capacity) separately, and made two sets, one set of sub-samples was spiked with known concentrations of standard solution of Se, while the other set were treated non-spiked. 20ml of ultrapure water (18Ω) was added to each flask, the mortar and pestle were rinsed with equal

amount of water and added to beakers with samples and properly stirred. The beakers with samples were covered and heated on an electric hot plate at 60 ± 10 ˚C for 20mins.

2.2.7.2.1 TOTAL SELENIUM DETERMINATION

For the determination of total selenium (SeT), 2 g duplicate of each herb and treatments were weighed in crucible dishes. Samples were crushed with a pestle. A total of 5ml of freshly prepared mixture of concentrated HCl and HNO3 (1:2 ratio) were used rinse the pestle and added into each dish. The dishes were placed over a Bunsen burner and then heated for 5mins in a fume cupboard. After decomposition of organic matrixes, the mixes were diluted in de-ionised water and filtered using a filter paper (Whatman no.2) and made up to 10ml with de-ionised water.

2.2.7.3 SAMPLE ANALYSIS

The extractible selenium content of aqueous extracts of herbs was determined according to Kolachi *et al.* (2010) with slight modifications. A series of standard solutions of selenium were prepared by diluting a certified standard solution of 1g/L Fluka (UK). 20µg of Mg $(NO_3)_2$ was prepared from a stock standard. 20 µg of Nickel was prepared from 99.99% nickel. To avoid contamination, all glass and plastic wares were washed and kept in 10% v/v HNO and rinsed several times with ultrapure water (18Ω) before use.

Instrumentation

Water extracts of herbs were analysed using the Perkin-Elmer Analyst 800 atomic absorption spectrometer (Norwalk CT, USA) equipped with HGA graphite furnance and deuterium background corrector. Perkin-Elmer pyrolytic-coated graphite tubes with a platform were used. Sample volumes of 10µl and 10µl of mixture of 0.010mg Mg $(NO_3)_2$ and nickel nitrate as matrix modifiers were injected at temperature of 20 ˚C into the furnance with the Perkin-Elmer AS-800 autosampler. A selenium hallow-cathode lamp was used as radiation source at 196.0 nm wavelength, slit width of 2.0 nm and lamp current of 10 mA. 99.99% pure argon was used as purge gas at a flow rate of 250 ml/min. The graphite furnance heating program was set at different steps of drying 1, drying 2; ashing, atomisation and cleaning at temperature range (˚C), ramp and hold time (seconds) of 110/1-30, 130/15-30, 1300/10-20, 1900/0-5 and 2450/1-3, were used respectively. It must be noted during atomisation there was no flow of argon.

Calibration and statistical analysis

The concentrations were obtained from a calibration graph after correction of absorbance from reagent blank signals. A blank sample (without extracts or standards) was run throughout the whole procedure. Calibration and standard graphs were obtained for selenium. The linear range of the standard curve was obtained up to 60 µg/L. A good correlation was obtained (R = 0.97).

Analysis at every point from each experiment was carried out in triplicates. Means and standard deviations were calculated from replicates. Quantification was carried out using the correlation coefficient "R" of the line of best fit from excel plots of standard concentrations as required. P-values less than 0.05 were considered statistically significant.

2.2.8 PHYTIC ACID DETERMINATION

The phytic acid in unprocessed products mainly appears as inositol hexaphosphate (IP6) or in its salt form as phytate. During extraction and determination of phytic acid, the amount in analysed food materials varies and depends on the extraction and analytical methods employed. Due to its saturated rings, phytic acid does not absorb with UV or visible region light. Therefore many researchers have reported on the identification and quantification of phytic acid from grains, creal products, biological and urine samples employing both chromatographic (Graf and Dintzis, 1982; Lehrfeld and Morris, 1992; Talamond, *et al*., 2000, Talamond, *et al*., 1998) and spectrophotometric methods (AOAC, 1990; Saad, *et al*., 2011; Park, *et al*., 2006) and the effects of processes on phytic acid concentration (Tajoddine, *et al*., 2011). Other methods employed in the determination of phytic acid content of food materials involves the use of its product of hydrolysis. These include phosphate or inositol which can be determined through gas chromatography and mass spectrometry. However, these need to be derivatised.

In this thesis, phytic acid content of extracts was identified with the UV spectrophotometer using the Wade reagent using the AOAC method. An effect of different processes on the phytic acid content of herbs was also investigated. Therefore, analysis was carried out in fresh, frozen (blanched and un-blanched).

2.2.8.1 ANALYSIS OF PHYTIC ACID CONTENT OF SAMPLES

1g of fresh potted and frozen plant samples were extracted with mortar and pestle using 15 ml 5% H_2SO_4 ph 0.6 as described by Norazalina Saad, et al. (2011) with slight modification. The

extraction was carried out at room temperature for10mins. The sample mixes were further sonicated for 30mins and centrifuged at 2000g for 20mins. The supernatants were decanted and the residue further rinsed with extraction solvent.

Phytic acid levels in herbal plants were determined according to the AOAC method as modified by Latta and Eskin (1980) with slight modification. A chromatographic column (0.7 cm x 15cm) containing 0.5g of an anion-exchange resin (100-200 mesh, chloride form; AG-X4) was equilibrated with 0.7M NaCl. The column was washed with distilled water. The supernatant of the sample mix and NaEDTA-NaOH solution was passed through the anion exchange column. The column was washed with distilled water and 0.1M NaCl to remove inorganic phosphorous. Retained phytic acid was eluted with 0.7M NaCl.

2.2.8.2 SAMPLE ANALYSIS

Phytic acid content of extracts was determined spectrophotometrically by reading absorbance at 500nm which reduces with the disappearance of purple colour from Wade reagent. Phytic acid concentration was calculated from the equation of thr graph prepared from standard solutions containing pure phytic acid from rice. Stock standard solution of 2mM of phytic acid was prepared using deionised water and standard solutions were prepared to appropriate concentrations (10µM-80µM) via dilutions of the stock solution.

Wade reagent (0.03% $FeCl_2$.$6H_20$ and 0.3% sulfosalicyclic acid in distilled water) was added to an aliquote of the extract and centrifuged at 3500rpm for 5mins. The absorbance of extract was read in triplicates at 500nm with a UV spectrophotometer using water as blank. Phytic acid concentration was calculated from the equation of graph prepared from standard solution containing pure phytic acid from rice.

2.2.9 INVITRO EVALUATION OF THE ROLE OF HERB EXTRACTS IN THE MANAGEMENT OF POSTPRANDIAL HYPERGLYCEMIA AND HYPERTENSION

In the control of postprandial hyperglycemia the best therapeutic approach is to reduce the absorption of glucose by the inhibition of key carbohydrate hydrolyzing enzymes in the digestive tract (Kwon *et al*, 2006). These enzymes include α-amylase and α-glucosidase. They catalyse the breakdown of oligo and disaccharides to monosaccharides such as glucose which is easily utilized by the body (Nikavar *et al*, 2008)

2.2.9.1 ALPHA AMYLASE ASSAY

Several *in vitro* assays have been carried out on α-amylase inhibition by herb extracts (Kwon *et al*, 2006; Nikavar *et al*, 2008; Wongsa *et al*, 2011). These assays are carried out *in vitro* using the porcine pancreatic α-amylase which is closely related to human pancreatic α-amylase. The ability of herb extracts to inhibit the activity of α-amylase is measured spectrophotometrically at 540nm by monitoring the colour difference between the enzyme/substrate (starch) reaction mixture in the absence of herb extract (control) and enzyme/substrate (starch) reaction mixture in the presence of herb extract. 3,5-dinitrosalicylic acid (DNSA) is used as colour indicator.

Another assay method for the inhibition of pancreatic α-amylase is the starch-iodine colour assay (Xiao *et al*, 2006; Sudha *et al*, 2011). In this assay method, the reaction mixture is the same as in the method mentioned above; however instead of DNSA, iodine reagent is used as the colour indicator. The colour change is noted at an absorbance of 620nm. In this assay, a dark-blue colour indicates the presence of starch, a yellow colour the absence of starch and a brownish colour partially degraded starch.

2.2.9.1.1 DETERMINATION OF THE ALPHA AMYLASE INHIBITION BY SAMPLES

The α-amylase inhibitory activity assay was performed by the methods of Tingting et al (2011) with slight modification. A total volume of 200µl mixture containing 100µl of each extract or 100µl phosphate buffer (no inhibition) and 100µl of α-amylase solution (0.5mg in 1ml 0.02M sodium phosphate buffer, pH 6.9 with 0.006M sodium chloride) were incubated at 25 ˚C for 10 min. After pre-incubation, 100µl of 1% starch solution dissolved in 0.02M sodium phosphate buffer was added and the reaction mixture incubated for another 10 min. After incubation, a volume of 200µl DNSA solution (3,5-dinitrosalicylic acid, 90mM) was added into the reaction mixture and tubes maintained in a boiling water bath for 5min. The tubes were then cooled to room temperature and 4ml distilled water was added. The absorbance of the mixture was measured at 540nm and the inhibitory activity of the extracts was calculated by;

$$\text{Inhibition activity (\%)} = \frac{\text{AB540nm control} - \text{AB540nm sample}}{\text{AB540nm control}} \times 100$$

2.2.9.2 ALPHA GLUCOSIDASE ASSAY

Alpha glucosidase inhibitory activity was performed as described by Kwon, *et al*. (2006) with slight modifications. 50µl of sample solution and 100µl of 0.1M phosphate buffer (pH 6.9) containing α-glucosidase solution (0.5U/ml) were incubated in 96 well plates at 25˚C for 10 minutes. After preincubation, 50µl of 5mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1M phosphate buffer (pH 6.9) was added to each well at timed intervals. Using a 96-well plate, the reaction mixtures were incubated at 25˚C for 5 minutes. Before and after incubation, absorbance readings were recorded at 405nm and compared to a control which had 50µl of buffer solution in place of the extract by micro-array reader (FLUOstar Optima, bmg Labtech). The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows:

$$\text{Inhibition activity (\%)} = \frac{\Delta\text{AB405nm control} - \Delta\text{AB405nm sample}}{\Delta\text{AB405nm control}} \times 100$$

The IC_{50} value of the extract, which is defined as the concentration of the extract that will bring about 50% inhibition of α-glucosidase under the stated assay conditions, was then determined. In the case of significant inhibition, for this research, IC_{50} values were determined using the linear regression dose response equation with the variable slope. All results are means of triplicate assays.

Kinetics of inhibition against α-glucosidase

The plant extract with the best α-glucosidase inhibition activity and the inhibition mode was determined by increasing the concentration [S] using PNPG (p-nitrophenylα-D-glucopyranoside) as substrate in the presence and absence of water extracts of herbs of different concentrations. The concentrations of substrates used were 1,2,3,4, 5, 6 mM of PNPG at two different concentrations of plant extract.

The enzyme reaction was carried out as described above. All assays were carried out in triplicates. Mode of inhibition type was determined by the Lineweaver-Burk plots analysis of data which were calculated from the results according to the Michaelis-Menten kinetics. The K_i values which are the experimental enzyme-inhibitor dissociation constant can be determined from the graph or calculated theoretically using equations depending on the particular experimental mode of inhibition.

2.2.10 ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITION ASSAY

There are a number of methods used in ACE inhibition assays. These include spectrophotometric (Jimsheena and Gowda 2009), fluorometric (Balasuriya and Rupasinghe 2011), high-performance liquid chromoatography (Wu *et al*, 2002; Kwon *et al*., 2006; Tingting *et al*., 2011) and capillary electrophoresis methods (Zhang e*t al*., 2000).

Two commonly used substrates for spectrophotometric and HPLC analysis are hippuryl-histidyl-Leucine (HHL) and N-(3[2-furyl]acryloyl-phenylala glycy L-glycine (FAPGG).

With the HPLC method using HHL as a substrate, the ACE inhibition assay is based on the hydrolysis of synthetic HHL as described by Wu *et al* (2002) and sometimes slightly modified (Wang *et al*., 2013). The released HA, histidine leucine (HL) and sometimes un-hydrolysed HHL separated by reversed-phase HPLC (Wu *et al.* 2002; Kwon *et al*., 2006; Tingting *et al*., 2011; Wang *et al*., 2013) is directly proportional to the ACE inhibition activity.

In the fluorimetric assay the fluorescence of a fluorimetric adduct (o-phthaladehyde) is used and fluorescence of reaction mixture is measured at excitement of 350nm and emission of 500nm (Balasuriya and Rupasinghe 2011).

2.2.10.1 DETERMINATION OF THE ACE INHIBITION ABILITY OF SAMPLES

ACE inhibition was assayed by a method modified by Kwon *et al.,* (2006). The substrate hippuryl-histidyl-leucine (HHL) and the enzyme ACE-I from rabbit lung (EC3.4.15.1) were used. 50 μL of sample extracts were incubated with 100 μL of 1 M NaCl-borate buffer (pH 8.3) containing 2 mU of ACE-I solution at 37 °C for 10 min. After preincubation, 100 μL of a 5mU substrate (HHL) solution was added to the reaction mixture. Test solutions were incubated at 37 °C for 1 h. The reaction was stopped with 150 μL of 0.5 N HCl. Five μL of the sample was injected in a highperformance liquid chromatography (HPLC) apparatus (Agilent 1100 series equipped with autosampler and DAD 1100 diode array detector, Agilent Technologies, Palo Alto, CA). The solvents used for gradient were (1) 10 mM phosphoric acid (pH 2.5) and (2) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% for 5 min and then was decreased to 0% for the next 5 min (total run time, 18 min). The analytical column used was an Agilent Nucleosil 100-5C18, 250 mm × 4.6 mm inside diameter, with packing material of 5 μm particle size at a flow rate of 1 mL/minute at ambient temperature. During each run, the absorbance was recorded at 228 nm, and the chromatogram was integrated using the Agilent Chemstation (Agilent Technologies) enhanced

integrator for detection of liberated hippuric acid (A). Hippuric acid standard was used to calibrate the standard curve and retention time. The percentage inhibition was calculated by:

$$\% \text{ inhibition} = \frac{(A\text{control} - A\text{extract})}{(A\text{control} - A\text{blank})} \times 100$$

2.2.11 DETERMINATION OF PREBIOTIC FUNCTION OF HERB EXTRACTS

A series of *in vitro* experiments were conducted to investigate the impact of aqueous extracts on the growth of two strains of lactic acid bacteria employing the method of Molan *et al.* (2008) with some modifications. Pure cultures of *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* were obtained from the culture collection held by the Public Health Culture Collection, England. These strains were grown to stationary phase in Mann-Rogosa-Sharpe (MRS) medium and then 1% (v/v) inoculums from these cultures were introduced into 2 ml of fresh MRS broth (Oxoid, England) containing different concentrations of the herb extracts. The extracts were first filter sterilised and added to MRS broths at 10% and 20% (v/v). The tubes were incubated at 37 ˚C for 48 h and at the end of the incubation period, the broths from control and extract-containing incubations were serially diluted 10- fold in fresh MRS broth and then 100 µl aliquots of each dilution were spread in duplicate on the surface of the plates containing MRS agar and incubated at 37 ˚C for 72 h for enumeration of *L. rhamnosus*. For *B. bifidum*, 0.05% cysteine hydrochloride was added to MRS broth. The MRS agar plates were incubated in an anaerobic incubator at 37 ˚C for 72 h. Growth of the strains was monitored by simple plate counting. Controls included a positive control (medium + bacteria) and negative control (medium only) containing 10 and 20% (v/v) sterile distilled water. Colony populations for each bacterial group were expressed as log colony forming units per ml (cfu/ml) medium. Data are analysed using T-test and the level of significance set at $P < 0.05$.

2.3 STATISTICAL ANALYSIS

Unless otherwise stated, all experiments were performed in triplicate. Means and standard deviations were calculated from replicates. Quantification of compounds was carried out using the correlation coefficient "R" of the line of best fit from a Microsoft Excel plot. Furthermore, with Microsoft Excel, one-way analysis of variance (ANOVA) was used to analyse mean

differences between samples, and a post-hoc t-test was performed to determine differences between individual groups. P values less than 0.05 were considered statistically significant.

CHAPTER 3

FUNCTIONAL CONTITUENTS OF HERBS- EFFECTS OF FREEZING AND BLANCHING ON THE FUNCTIONAL CONSTITUENTS OF *LAMICEAE* HERBS

3.1 INTRODUCTION

In this thesis, the functional constituents of plant origin analysed include those that have been either reported or assumed to be linked to their functional properties. The lamiceae herbs of mint, thyme and basil have been studied as fresh, un-blanched and blanched frozen preparations. The constituents examined include the phenolics determined as the total phenolic content (TPC), ascorbic acid content (covering the total, reduced and oxidized forms), selenium and phytic acid.

Phenolic compounds are generally known to be the main antioxidant compounds of plant origin. Phenolic compounds can be classified into at least 10 different classes, however the most prominent and significant ones obtained from our diets are simple phenols, phenolic acids, hydroxycinnamic acids, coumarins and flavonoids. However, flavonoids are the most abundant class with almost 6000 identified compounds (Jaganath and Crozier, 2010).

Phenolic compounds are mainly produced through the shikimate/phenylpropanoid pathway as a response to biotic and abiotic factors such as temperature.

Ascorbic acid is a naturally occurring organic compound derived from glucose which forms one of the most important molecules in human diet. Ascorbic is most highly susceptible to degradation and oxidation by chemicals and enzymes during processing, cooking and storage of produce. The ratio of ascorbic acid and dehydroascorbic acid (AA/DHA) is sometimes regarded as an indicator of the redox state of an organism therefore it has been deemed necessary to simultaneously determine the concentration of AA and DHA (Gillespie and Ainsworth, 2007).

Selenium is regarded as an essential nutrient for humans because it is an essential component of several major metabolic pathways, including thyroid hormone metabolism, antioxidant defence systems and immune function. It has been recognised as an integral component of different enzymes such as thioredoxin reductase and glutathione peroxidase, which participate in the antioxidant protection of cells (Birringa *et al*., 2002).

Phytic acid (myoinositol hexa-phosphoric acid, IP6) is the major phosphorus storage compound of most seeds and cereal grains, it may account for more than 70% of the total phosphorus. Excess phytic acid has a strong ability to chelate multivalent metal ions, such as copper, zinc, calcium and iron at physiological pH leading to the formation of insoluble complexes (Graf, 1986). Its reaction with Fe leads to the formation of iron-phytate chelate which is totally inert in the Fenton reaction. The ability of phytic acid to form this complex makes Fe unavailable for hydroxyl radical formation - a reaction which exhibits its ability to act as an antioxidant. Due to its antioxidative potential, it has aroused great interest as a potential food preservative and therapy for pathological diseases caused by free radicals (Stodolak, *et al*., 2007; Harbach *et al*., 2007; Soares *et al*., 2004; Lee and Hendricks, 1995).

Although phytic acid is said to be water soluble and heat labile, there has not been any report on the effects of freezing on phytic acid.

AIMS AND OBJECTIVES

The aims and objectives of this result chapter include the following;

1. To determine phenolic content (measured as their total phenolic content), ascorbic acid content (reduced, oxidized and total ascorbic acid), selenium and phytic acid content of chosen herbs across treatments.
2. To use Spectrophotmeteric methods to determine the TPC, ascorbic acid, selenium and phytic acid content of selected herbs.

3.1.1 RESULTS

The antioxidant constituents of herbs were determined as described in Chapter 2; TPC (section 2.2.4), ascorbic acid (section 2.2.6), selenium (section 2.2.7) and phytic acid (section 2.2.8).

The results of the effects of blanching and freezing on the total phenolic content, ascorbic acid content, selenium and phytic acid content of herbs varied among treatments and herbs.

3.1.1.1 TOTAL PHENOLIC CONTENT (TPC)

The total phenolic content of herbs was determined as described in Chapter 2 (section 2.2.4). The TPC of herbs are expressed as milligram Gallic acid equivalent per gram dry weight of herb (mg GAE/g dw herb).

Results of the total phenolic content of herbs across all treatments and extraction solvents is summarised in Table 3.1. The TPC of selected herbs differed/varied among herbs depending on the extraction solvent and treatment given to herbs. Generally, the water extracts of herbs gave a significantly lower TPC value than methanol extract. However, methanol extracts of fresh mint possessed the highest TPC across all treatments and solvent extractions (284.20mg GAE/g dw herb).

Herbs	Extraction solvent	Treatment (± standard deviation)		
		Fresh (mg GAE/g dw herb)	Blanched frozen (mg GAE/g dw herb)	Un-blanched frozen (mg GAE/g dw herb)
Mint	Water Methanol	37.98 ± 3.25 284.20 ± 50.25	25.11 ± 7.02 159.18 ± 27.34	40.83 ± 9.01 211.53 ± 92.02
Thyme	Water Methanol	12.61 ± 0.97 87.16 ± 14.19	7.35 ± 2.11 84.01 ± 17.03	13.77 ± 1.74 101.62 ± 15.23
Basil	Water Methanol	4.66 ± 0.54 98.09 ± 20.02	12.43 ± 2.02 182.73 ± 20.87	17.71 ± 3.46 205.13 ± 50.32

Table 3. 1Results of total phenolic content of different treatments of herbs. Results are means of three different readings (± standard deviation).

Figure 3.1 shows a representation of TPC of water and methanol extracts of herbs at different treatments. For mint, TPC values of methanol extracts are in the order, fresh > un-blanched frozen > blanched frozen mint. A single factor ANOVA using excel showed a significant difference between the TPC of sample extracts (P = 4.18E-09). A further post-hoc t-test showed that, the TPC values of fresh extracts is significantly higher than the extracts of frozen mint (P = 2.29E-05 for blanched, and P = 1.81E-07 for unblanched). Furthermore, TPC of un-blanched frozen mint is significantly (P = 8.54E-05) higher than that of blanched frozen herbs.

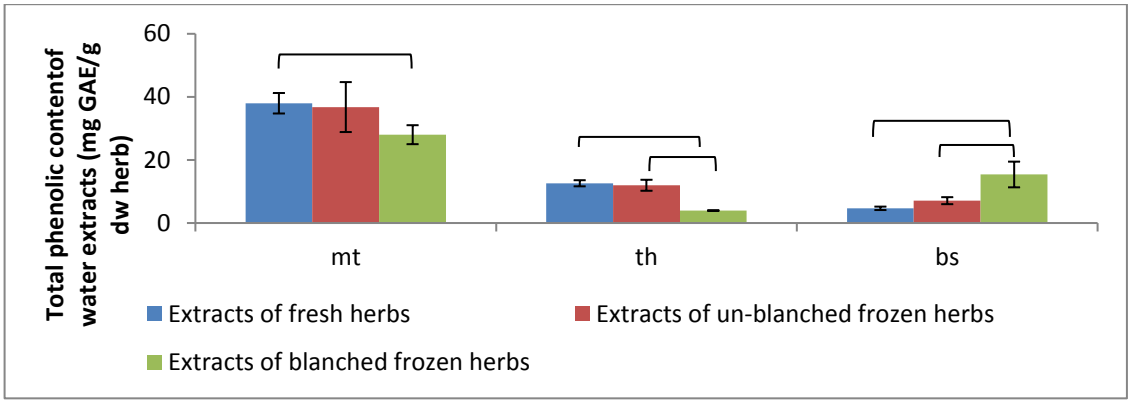
In contrast to methanol extracts of mint, the TPC values of water extracts of mint are in the order, un-blanched frozen > fresh > blanched frozen mint. An ANOVA of all the extracts of different treatment showed a significant difference (P = 2.7E-04) between the TPC values of all extracts. However, further post hoc t test showed that there is no significant difference (P =0.29) between the TPC of water extracts of fresh and blanched frozen extracts. However, the TPC of un-blanched frozen is significantly higher than that of both fresh (P =7.4E-04) and blanched frozen (P = 1.81E-04) mint.

Results obtained with thyme generally showed that the least TPC values were obtained with blanched frozen thyme extracts compared to fresh and un-blanched frozen thyme. For methanol extracts, a single factor ANOVA showed a significant difference (P = 0.0005) between the TPC values of all extracts (Fresh, blanched and un-blanched frozen). However, there was no significant difference (P = 0.25) between the extracts of blanched and un-blanched frozen extracts. Results of TPC of water extracts showed that the highest TPC value was obtained with the un-blanched frozen compared to fresh and blanched frozen samples. Although an ANOVA of TPC values of extracts showed a significant difference (P = 7.4E-06) between all extracts, there is no significant difference (P = 0.86) between results of extracts of fresh and blanched frozen thyme extracts. However, there is a significant difference between un-blanched frozen and other treatments (P = 5.14E-05 for fresh, and P = 5.7E-05 for un-blanched frozen).

The results of TPC values of basil showed that the highest values were obtained with un-blanched frozen basil while the least values were obtained with extracts of fresh basil for both water and methanol extracts. An ANOVA between methanol extracts showed that the TPC values of extracts are significantly different (P = 1.7E-12). Further post hoc t-test showed that results of blanched frozen extracts are significantly higher than fresh and un-blanched frozen extracts (P = 2.14E-10, and P = 0.02, respectively). Furthermore, blanched frozen samples had a significantly (P = 3.08E09) higher TPC compared to fresh samples. In contrast to results of TPC value of methanolic extracts, results TPC values of water extracts of basil showed that highest value is obtained with blanched frozen extracts and the least with fresh extracts. An ANOVA of TPC values of water extracts showed a significant difference (P = 3.78E-05) between values of all extracts, however, a post hoc t-test showed that there is no significant difference (P = 0.71) between the TPC values of blanched and un-blanched frozen basil extracts. Furthermore, the TPC values of both blanched and un-blanched extracts are

significantly higher than those of fresh basil extracts (P = 4.22E04 for blanched frozen, and P = 2.18E-07 for un-blanched frozen).

(a)



(b)

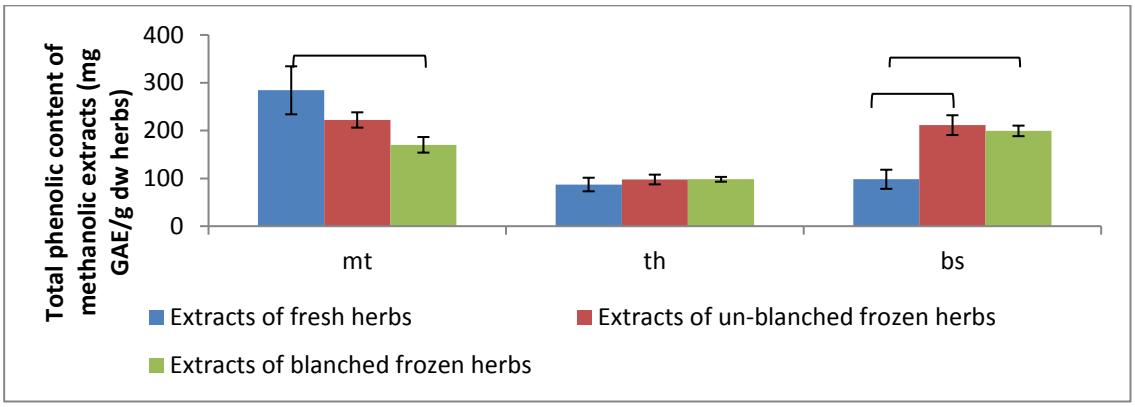


Figure 3.1 Representation showing the effects of blanching and freezing on TPC value of (a) Water and (b) Methanol extracts of fresh, un-blanched and blanched frozen herbs. Mint (mt); Thyme (th) and Basil (bs). Values denoted with ⊓ are significantly different (P < 0.05)

3.1.1.2TOTAL, REDUCED AND OXIDIZED ASCORBIC ACID CONTENT

Table 3.2 shows the results of total, reduced and dehydroascorbic acid (TAA, RAA and DHA respectively) for fresh, blanched and un-blanched frozen herbs. The DHA is calculated as the difference between each of the triplicate readings of TAA and RAA (TAA - RAA= DHA).

For mint extracts, the highest TAA value is obtained with the fresh mint extracts (25.57 mg TAA/g dw), while the least is obtained with the extracts of un-blanched frozen mint (8.02mg TAA/g dw herb). A single factor ANOVA using excel showed a significant difference (P = 1.0E-12) between all extracts of mint. A further post hoc t test also showed a significant difference between TAA values of extracts of fresh and frozen herbs (P = 2.84E-08 for

blanched, and P = 2.28-11 for un-blanched). Therefore, freezing of fresh mint without blanching (un-blanched frozen) led to an approximately 69% loss of its TAA content while blanching prior to freezing led to an approximately 44% loss of TAA content. Furthermore, comparing un-blanched and blanched frozen mint, there is a significant (P = 2.5E-04) 44% loss in TAA content in un-blanched frozen mint.

Results of the RAA showed that extracts of un-blanched frozen mint had the lowest RAA value. Although, an ANOVA showed a significant difference (P = 1.28E-09) between all mint extracts, a post hoc t-test show that there is no significant difference (P = 0.67) between the RAA contents of extracts of fresh and blanched frozen mint. However, freezing without blanching (un-blanched frozen) of mint showed to have significantly reduced the RAA content both (P = 3.82E-07) fresh and (P = 1.56E-09) blanched frozen by about approximately 65% and 66%, respectively.

For the DHA content of mint, an ANOVA of all extracts showed a significant difference (P = 3.26E-12) between all extracts. A further post hoc t-test showed that the DHA content of fresh mint extract is significantly higher than the DHA content of extracts of both blanched and un-blanched frozen mint. Hence, comparing the effects of freezing on the DHA content of fresh mint, freezing without blanching (un-blanched frozen) significantly (P = 3.95E-09) reduced the DHA content of fresh mint by about 72% while blanching prior to freezing showed to significantly (P 2.03E-09) reduce the DHA content of fresh mint by about 84%. Furthermore, comparing the DHA content of blanched frozen to that of un-blanched frozen, blanching prior to freezing seem to significantly (P = 2.27E-03) reduced the DHA content of frozen mint by about 44%.

Herbs	Ascorbic acid assay	Fresh herbs	Frozen herbs	
			Blanched	Un-blanched
Mint	TAA	25.57 ± 1.22	14.42 ± 2.09	8.02 ± 0.48
	RAA	12.43 ± 1.93	12.69 ± 0.66	4.33 ± 0.32
	DHA	13.09 ± 0.73	2.06 ± 0.80	3.69 ± 0.13
Thyme	TAA	22.83 ± 2.68	13.28 ± 1.97	15.98 ± 3.92
	RAA	17.50 ± 2.92	11.77 ± 1.24	6.71 ± 1.13
	DHA	5.33 ± 0.20	1.15 ± 0.60	9.27 ± 2.3
Basil	TAA	22.11 ± 0.45	12.03 ± 1.52	16.14 ± 3.15
	RAA	14.43 ± 3.78	10.83 ± 2.11	5.07 ± 2.21
	DHA	7.68 ± 2.72	1.20 ± 0.48	11.07 ± 0.77

Table 3. 2 Summary of the total reduced and oxidized ascorbic acid in herbs. MT (mint), BS (Basil), TH (Thyme). TAA (total ascorbic acid), RAA (reduced ascorbic acid), DHA (dehydroascorbic acid). All results are mean of triplicate assays. All results are expressed as mg/g dry weight (± standard deviation)

The ANOVA of results of the ascorbic acid content of thyme shows a significant difference (P = 3.6E-05) between the TAA content of fresh, and frozen (un-blanched and blanched) thyme. A post hoc t-test showed a significant loss of 30% (P =2.004E-04), and 42% (P = 2.4E-03) TAA content of thyme when samples are frozen (un-blanched and blanched frozen respectively) compared to fresh thyme. However, there is no significant (P = 0.16) loss of TAA content between un-blanched and blanched frozen.

The results of RAA content of thyme showed a significant difference (P = 2.80E-08) between the RAA content of fresh, and frozen (un-blanched and blanched) with un-blanched frozen thyme having the least. Furthermore, a post hoc t-test showed a significant loss of approximately 62% (P = 1.16E-06) and 33% (P = 3.86E-05) in RAA content of thyme when frozen (un-blanched and blanched respectively) compared to fresh thyme.

Results of the DHA content of thyme showed a significant (P = 1.3E-05) difference between the results of fresh, and frozen (blanched and un-blanched) samples. A further post hoc test showed that there is a significant 43% (P = 1.11E-04) and 88% (P = 2.28E-06) more DHA in un-blanched frozen than in fresh and blanched frozen thyme respectively.

For basil, ANOVA of the results data obtained showed that there is significant difference (P = 7.02E-07) in the TAA contents between the extracts of fresh, and frozen (blanched and un-blanched) samples. Further post hoc t-test between result data of TAA of fresh and un-blanched frozen showed that freezing significantly reduced the TAA content of fresh sample by approximately 27% (P = 4.78E-04) and 46% (P = 7.9E-09) for un-blanched and blanched frozen respectively). However, there was a slight significance (P = 0.021) difference between the TAA content of un-blanched and blanched samples.

An ANOVA of results of RAA of basil showed a significant (P = 1.97E-06) difference between fresh and frozen (blanched and un-blanched) samples. A post hoc t-test of the values of RAA content of basil, shows that freezing without blanching (un-blanched frozen) of fresh samples led to a significant (P = 2.16E-05) reduction of RAA by approximately 65% while blanching prior to freezing led to a slightly significant (P = 0.011) reduction of 25%. Furthermore, freezing without blanching showed a significant (P =8.4E-05) loss of approximately 40% of RAA compared to blanching prior to freezing.

For DHA content of basil, the highest value was obtained with un-blanched frozen sample and the least value was obtained with blanched frozen samples. An anova of results showed a significant (P = 1.35E-10) difference between fresh and frozen (un-blanched and blanched) samples. A post hoc test showed 84% (P = 9.04E-07) and 89% (P = 3.93E-10) low DHA value of blanched frozen samples compared to fresh, and un-blanched frozen samples respectively.

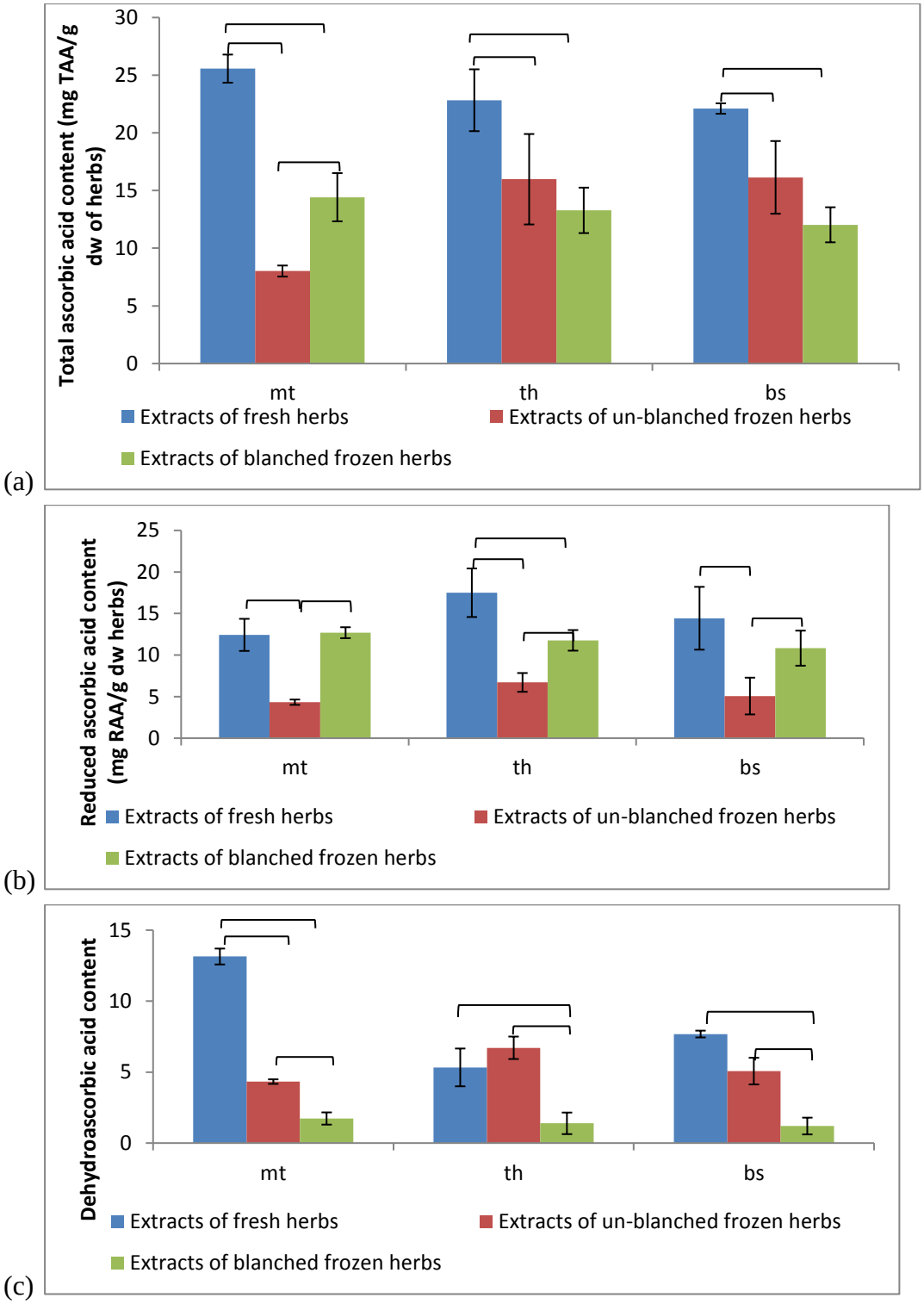


Figure 3.2 Microsoft Excel plot of a representation of (a) Total ascorbic acid content; (b) Reduced ascorbic acid and (c) Dehydroascorbic acid of herbs; Mt (mint), Th (Thyme), Bs (Basil). Values denoted with ⌈ are significantly different (P< 0.05)

3.1.1.3SELENIUM CONTENT

Results of both total and extractible selenium are presented in Table 3.3 Selenium contents were calculated from calibration curve (R = 0.97) giving a linear range of 0.1 - 0.5 µg/L (See Appendix).

Herbs	Se_{Total}			$Se_{Aqueous}$		
	Fresh	UBfz	Bf	Fresh	UBfz	Bf
Mint	0.40 ± 0.017	0.21 ± 0.01	0.14 ± 0.037	0.021 ± 0.003	0.08 ± 0.01	0.053 ± 0.017
Thyme	0.14 ± 0.021	0.03±0.002	0.03 ± 0.001	0.010 ± 0.001	0.02 ± 0.003	0.014 ± 0.004
Basil	0.05 ± 0.002	0.09 ± 0.01	0.04 ± 0.011	0.013 ± 0.004	0.043 ± 0.013	0.008 ± 0.002

Table 3. 3. Determination of total and water extractible selenium (Se_T and $Se_{Aqueous}$) content (µg/g dw) of fresh, un-blanched frozen (Ubfz) and Blanched frozen (Bfz) herbs. All results are mean of triplicate assays. All results are expressed as mg/g dry weight (± standard deviation)

Table 3.3 presents the means of standard deviation for both Se_T and $Se_{Aqueous}$ of different herb treatments (fresh, un-blanched frozen and blanched frozen herbs). The Se_T of herbs for all treatments ranged from 0.03 to 0.40 µg/g dry weigh of herbs while $Se_{Aqueous}$ ranged from 0.008 to 0.053 µg/g dry weigh of herbs. It was observed that 5 - 26 %, 20 - 50% and 38 - 70% of Se was extracted into the medium for fresh, blanched frozen and un-blanched frozen herbs respectively. Hence, generally the highest aqueous extractability was obtained from un-blanched frozen herbs while extracts from fresh herbs showed the least $Se_{Aqueous.}$ The ANOVA of $Se_{Aqueous.}$values obtained for mint showed a significant difference (P = 8.62E-05) between results of $Se_{Aqueous}$ of fresh, and frozen samples (blanched and un-blanched frozen). A post hoc test showed that fresh samples showed to be significantly the least compared to un-blanched (P = 3.46E-06) and blanched (P = 3.65E-03) frozen samples. However, there is slight significant difference (P = 0.03) between values of the $Se_{Aqueous}$ of un-blanched and blanched frozen samples.

Results of the selenium content of thyme showed that like mint, fresh thyme samples showed to have the least $Se_{Aqueous}$ content. However, unlike mint, un-blanched frozen thyme samples had the highest $Se_{Aqueous}$ value. An ANOVA of results showed that there is significant (P = 2.3E-03) difference between fresh and frozen (blanched and un-blanched) extracts of thyme. A post hoc test showed a significant difference between of fresh and frozen samples (P = 1.75E-

04 for un-blanched; P = 1.5E-03 for blanched). Furthermore, there is a significant (P = 9.49E-03) difference between blanched and un-blanched frozen samples.

Results of the $Se_{Aqueous}$ content of basil showed a significant (P =0.0001) difference between fresh and frozen (blanched and un-blanched) samples. Furthermore, un-blanched frozen samples had a significantly higher $Se_{Aqueous}$ compared to fresh (P = 1.3E-03) and blanched (P = 6.89E-06) frozen thyme.

Since extract of aqueous extracts are needed for further application in this thesis, only the result of extractable selenium content of herb extracts is represented graphically in Figure 3.3.

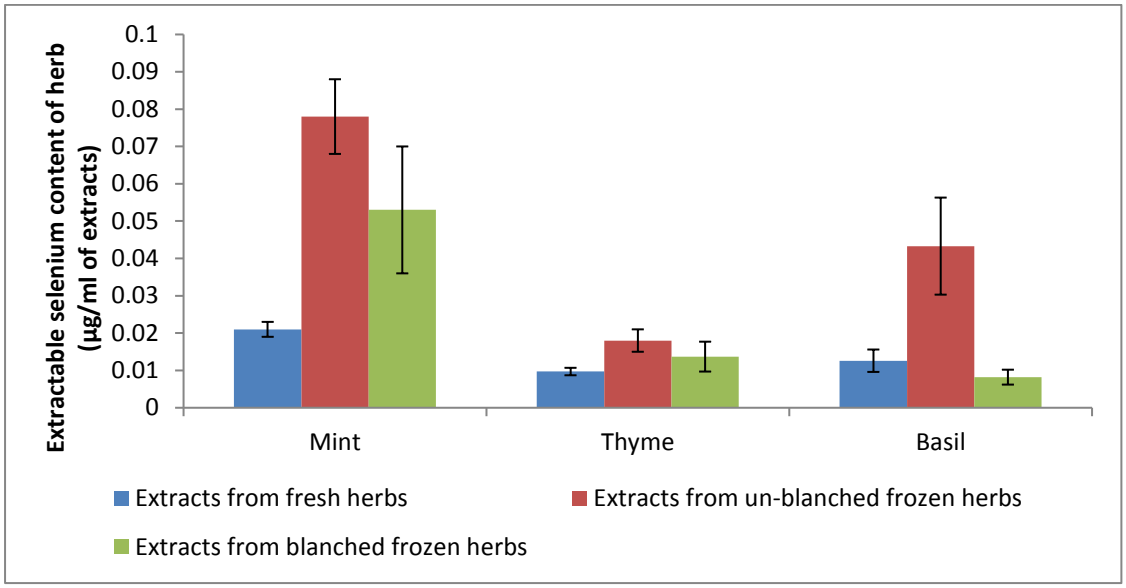


Figure 3.3 Representation of extractable selenium content of fresh herb extracts. Statistical analysis based on t-test. (P values < 0.05 are significantly different)

3.1.1.4 THE PHYTIC ACID CONTENT OF LAMAICEAE HERBS AND EFFECTS OF FREEZING/BLANCHING ON PHYTIC ACID CONTENT

Results showing the effect of freezing (un-blanched and blanched) of phytic acid content are presented on table 3.4 and Figure 3.4.

From the results on Table 3.4, fresh mint has the highest phytic acid content (54.82 ± 9.24 mg phytic acid/g dw herb) followed by basil (20.78 ± 3.07 mg phytic acid/g dw herb) and thyme (9.14 ± 0.98 mg phytic acid/g dw herb).

For mint, the least phytic acid content was obtained with blanched frozen herbs, while fresh samples had the highest. Furthermore, ANOVA of data obtained with mint extracts showed a

significant (P = 2.3E-10) difference between the phytic acid values of fresh, blanched and un-blanched samples. However, there was loss of approximately 62%, and 85% of phytic acid when fresh samples are frozen (un-blanched and blanched frozen samples respectively). Furthermore, blanching prior to freezing showed a significant loss of 62% of phytic acid.

Herbs	**Phytic acid content of herbs**		
	Fresh herbs	**Frozen herbs**	
		Blanched	**Un-blanched**
Mint	54.82 ± 9.24	8.06 ± 2.05	21.04 ± 3.02
Thyme	9.14 ± 0.98	3.76 ± 1.05	5.75 ± 0.93
Basil	20.78 ± 3.07	10.02 ± 1.42	19.39 ± 4.12

Table 3. 4. Summary of the phytic acid content of herbs. All results are mean of triplicate assays. Results are expressed as mg/g dry weight (± standard deviation)

Similarly, ANOVA of result data of thyme extracts showed that there is a significant (P = 1.12E-04) difference between the phytic acid content of fresh and frozen samples (un-blanched and blanched). Furthermore, a post hoc t-test showed that freezing of fresh samples without blanching (un-blanched frozen) significantly (P = 1.67E-03) reduced the phytic acid content of fresh by 37%. However, comparing the phytic acid content of blanched frozen samples to those of fresh samples, blanching prior to freezing seemed to significantly (P = 4.96E-04) reduce the phytic acid content of fresh by 59%. Comparing un-blanched to blanched frozen samples, there was a slight significant difference (P = 0.03) between the phytic acid content of both frozen samples.

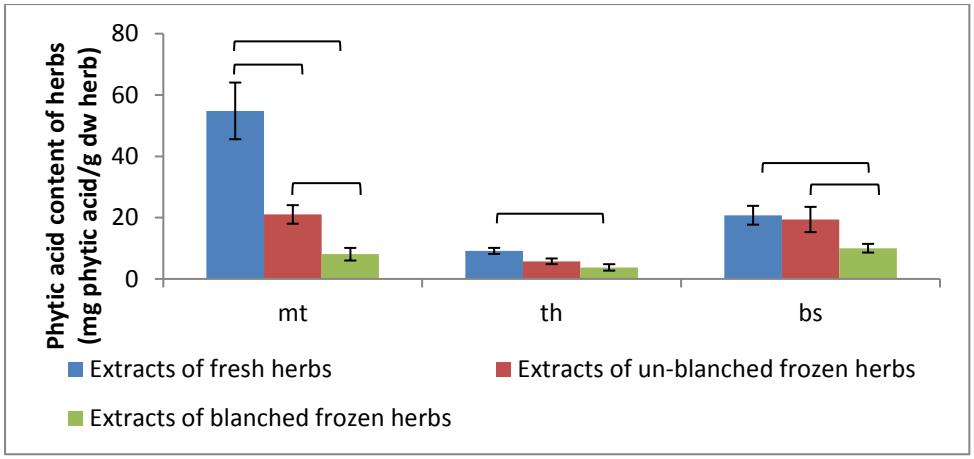


Figure 3.4. Microsoft Excel plot of a representation of the phytic acid content of herbs; Mt (Mint), Th (thyme), Bs (Basil). Values linked with ∩ are significantly different (P < 0.05)

For basil, ANOVA of the result data showed that there is significant (P = 3.4E-04) difference between the phytic acid content of fresh, and frozen (Un-blanched and blanched) samples. Furthermore, a post hoc t-test showed no significant (P = 0.7) difference between results of fresh and un-blanched frozen samples, that is, there is slight to insignificant reduction in the phytic acid content of fresh basil after freezing. However, there is significant difference between results of fresh and blanched samples (P = 6.96E-04), and blanched and un-blanched samples (P = 0.002). Hence, blanching prior to freezing significantly reduced the phytic acid content of both fresh, and un-blanched frozen samples by approximately 52%.

3.1.2 DISCUSSION

Phenolic compounds in vegetables are said to be present as soluble, combined and insoluble (bound) forms in plant cell wall. Losses incurred with blanched frozen herbs may be attributed to the increased surface area of tissues in contact with blanching water and high temperature which is likely to cause softening, disruption/breakdown of cell walls/cellular components (vacuoles and apoplast) which harbour phenolic compounds (Friedman, 1996; Hong and Ahn, 2005; Kalt, 2005). These subsequently may lead to the degradation or the decomposition of thermal labile phenolics such as catechin (Francisco, *et al*, 2010) and leaching out of easily soluble phenolics, hence the reduction of phenolic content (Crozier, *et al.*, 1997).

Furthermore, low TPC of un-blanched frozen herbs may be attributed to enzymatic activity which may lead to irreversible changes in phenolic compounds such as oxidation with

subsequent polymerisation, decomposition (Waterman and Mole 1994; Zhang and Hamauzu 2004) and formation of aglycons which have low/no reactivity with Folin Ciocalteu reagent. The high TPC value attained with water extracts of blanched frozen basil may be due to the disruption of plant cell wall by heat treatment coupled with freezing which allowed for solvent-compound interaction of the phenylpropanoids and flavonoids which accumulate in the central vacuoles, epidermal and sub-epidermal cells of leaves (Hutzler, *et al.*, 1998). Furthermore, blanching may have led to the liberation of cellular bound hydroxycinnamic acids of chlorogenic and p-coumeric acids.

In addition, extraction solvents are said to be the most important factors in studying phytochemical compounds such as phenolic compounds from medicinal and plant foods. Phenolic compounds which are polar compounds have been reported to be better extracted using polar solvents such as ethanol, methanol and acetone (Kylli, 2011) but water has been reported as having lower extractability (Tuberoso, *et al*, 2010). In this research, the low TPC value obtained with water maybe because extractions were carried out at room temperature. Sources have reported that dissolution process of phenolic compounds such as protocatechuic acid, Gallic acid and catechin in water is endergonic, exothermic and entropy dependant. Hence an increase in extraction temperature enhances the solubility of these phenolic compounds (protocatechuic acid, Gallic acid and catechin) in water (Srinivas, *et al*, 2010). However, alteration/variation of the ph has been reported to enhance extractability of phenolic compounds in water (Friedman and Jurgens, 2000). Furthermore, acidification has been reported to aid extractability of phenolic compounds in fruits (Kalt, *et al*, 2000, Vuthijumnok, 2013) and apple juice by enhancing the stability of some phenolic compounds (Friedman and Jurgens, 2000). However, enhancement/stability is reported to be also dependent on the structure of phenolic compounds.

The significant reduction of total ascorbic acid in extracts of blanched and or frozen herbs indicates either the leaching/denaturation of water-soluble, heat labile ascorbic acid during blanching and enzymatic degradation of ascorbic acid in the process of freezing. Similar results were reported by Munyaka *et. al.* (2010) and Raseetha *et al*. (2013).

Furthermore, lower DHA values of blanched herbs may be attributed to the heat inactivation of degradative enzyme ascorbic acid oxygenase (Munyaka, *et al*, 2010). Oxidation reactions may be induced by increased temperature, high pH, light, presence of oxygen or metals and enzymatic action (Novakova, *et al.*, 2008) giving rise to oxidized ascorbic acid or

dehydroascorbic acid (DHA). In many horticultural crops, DHA has been reported to represent less than 10% of total ascorbic acid. However, the DHA content of crops are predisposed to increase during storage (Wills *et al*., 1984) due to oxidative and enzymatic degradation by ascorbic acid oxygenase.

The effects of freezing on selenium content of herbs and its extractability has not been studied so far. However, freezing is known to lead to the formation of ice crystals which ruptures the cell membranes of vegetables leading to easy extractability of compounds by encouraging solvent permeability into the cell matrix. There have been varying and inconsistent reports from different studies on the effects of cooking/processing on selenium content of foods. Some studies have reported that usual cooking procedures do not result in the loss of selenium while some studies reported the volatization of selenium by cooking methods such as boiling, baking and grilling (Dumont *et al*., 2006; Sager, 2006).

The results of phytic acid content showed significant loss of phytic acid in extracts of blanched frozen herbs compared to fresh herbs. Although there is no research on the effects of blanching or freezing on the phytic acid content of herbs, loss in the phytic acid content of herbs may be attributed to heat treatments such as blanching which have been reported to bring about the reduction/hydrolysis/degradation of phytic acid either chemically or enzymatically.

The enzyme, phytase, when/if activated can bring about the hydrolysis of phytic acid in less harmful and easily digestable inositol compounds. Furthermore, phytic acid which is water soluble has been lost through leaching during water blanching of herbs (Gobbetti, *et al* 2005).

3.1.3 CONCLUSION

From the results it can be concluded that blanching and/or freezing can either cause an increase or reduction of total phenolic content of herbs. Furthermore, choice of extraction solvent affects the extractability of phenolic compounds from herbs. Hence to obtain maximum efficiency/outcome of herbs for food and nutraceutical use, the right treatment and extraction solvent should be highly considered. For instance, from the results it can be concluded that domestically, freezing without blanching mint, thyme and basil is the best treatment required

to obtain high levels of phenolic compounds. Furthermore, for nutraceuticals, for a better extractability and higher yield of phenolic compounds from both thyme and basil, methanol is the preferred extraction solvent for un-blanched frozen preparation.

Blanching and freezing of herbs caused significant reduction of phytic acid, selenium and ascorbic acid content of selected herbs. Blanching can also be regarded as a tool for the control of DHA formation in herbs during storage.

CHAPTER 4

EFFECTS OF DOMESTIC FREEZING (-20˚C) TEMPERATURE AND BLANCHING ON THE PHENOLIC ACID CONTENT AND ANTIOXIDANT PROPERTIES OF LAMIACEAE HERBS

4.1 INTRODUCTION

A natural antioxidant has been defined as a substance which when at low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substrate (Prior, et al 2004). The number of antioxidant components in plants makes it relatively difficult to separately measure or determine the effects of each individual component. However, several researchers have proposed different assays deemed "generally suitable" to determine the antioxidant capacity of complex structures such as plant extracts.

Antioxidant capacity of plant foods practically deals with the synergistic action of a wide variety of antioxidants such as vitamins C and E and polyphenols, carotenoids, terpenoids, Millard compounds and trace minerals (Ou *et al*., 2002). The antioxidant activity is the most widely researched health benefits of culinary herbs which can also help to delay/retard spoilage due to rancidity and microbial activity.

Among the many methods for the in vitro analysis of potential antioxidants in foodstuffs, there are those that measure the ability of the antioxidant to break the chain reaction of lipid peroxidation (Schleiser, Harwat, Bohm, and Bitsch, 2002; Roginsky and Lissi, 2005), those that absorb and neutralize free radicals (Osawa, 1999) and those that bind metal ions with the formation of a complex (Yoshida *et al*., 2003).

Total antioxidant capacity assays are characterised into two major mechanisms, the first is Hydrogen atom transfer (HAT) which measures the ability of an antioxidant to quench free radicals (peroxyl radical thermally generated from Azo compounds) by H-atom donation include the oxygen radical absorption capacity (ORAC), total radical absorption potential (TRAP; Wayner, et. al. 1985). These include Oxygen radical absorption capacity (ORAC) and the total peroxyl radical trapping antioxidant parameter (TRAP).

The second mechanism is the single electron transfer (SET). The SET antioxidant capacity mechanism detects the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals (Wright *et al*., 2001). Furthermore, it measures the capacity of an antioxidant to reduce an oxidant which changes colour when

reduced. These include Trolox equivalent antioxidant capacity (TEAC), copper reducing antioxidant capacity (CUPRAC) by Apak *et al*., (2004), Diphenyl, picryl hyradzyl scavenging capacity (DPPH) and the Ferric reducing antioxidant potential (FRAP) by Benzie and Strain (1996).

Utilization of one assay to reflect all antioxidant activity in a complex system is said to give an inaccurate result due to multiple reaction characteristics, reaction mechanisms and different phase localizations. Therefore, it has been suggested that more than one antioxidant assay be used to analyse the antioxidant content/capacity of a system (Prior *et al*., 2005).

Based on the limitations of antioxidant assays like TRAP and ABTS this research focused/employed three electron transfer antioxidant assays (FRAP, DPPH and CUPRAC) and a hydrogen atom transfer antioxidant assay (ORAC) for all total antioxidant activity assays.

Phenolic compounds are generally known to be the main antioxidant compounds of plant origin. Phenolic compounds can be classified into at least 10 different classes, however the most prominent and significant ones obtained from our diets are simple phenols, phenolic acids, hydroxycinnamic acids, coumarins and flavonoids. However, flavonoids are are the most abundant class with almost 6000 identified compounds (Jaganath and Crozier, 2010).

Phenolic compounds are mainly produced through the shikimate/phenylpropanoid pathway as a response to biotic and abiotic factors such as temperature.

The aims and objectives of this study were to:

1. Compare the different assayed *Lamiaceae* herbs with regards to their different antioxidant activities measured as their radical scavenging ability against DPPH, metal chelating abilities (FRAP and CUPRAC) and oxygen radical absorbance capacity (ORAC) and ascertain if domestic freezing (-20˚C) and blanching significantly influences these antioxidant abilities
2. Furthermore, determine the effects of freezing and blanching on some individual phenolic acid content of extracts of herbs;
3. Compare the antioxidant activities of different phenolics and total antioxidant content of herb extracts measured as DPPH scavenging ability and FRAP to those of different synthetic antioxdants and phenolic compounds.

4.2 RESULTS OF TOTAL ANTIOXIDANT ACTIVITY ASSAYS

The total antioxidant activity assays measured as the diphenyl hydrazyl picryl inhibition (DPPH), ferric reducing antioxidant potential (FRAP), Cupric reducing antioxidant capacity (CUPRAC) and oxygen radical absorption capacity (ORAC) were performed on fresh, un-blanched and blanched frozen herbs with water and methanol as described in Chapter 2 (Section 2.2.3). The antioxidant activities of herbs measured showed varying results.

Results of samples were corrected for their moisture contents by converting their individual fresh weights into dry weights on the basis of their respective moisture contents (Chapter 2; section 2.2.2). The dry weights were then used for the calculation of the total antioxidant activity measured as CUPRAC and ORAC.

4.2.1 RESULTS OF DPPH ASSAY

In the DPPH inhibition assay, the antioxidant activity was measured by recording the loss in the absorbance as DPPH radical received an electron or hydrogen radical from an antioxidant source (in this case mint, thyme and basil) with subsequent formation of a stable diamagnetic molecule (Juntachote and Berghofer, 2005).

The results of the DPPH inhibition (percentage inhibition) assay is summarised and represented in Table and Figure 4.1

Herbs	Extraction solvent	Treatment (± standard deviation)		
		Fresh (%)	Blanched frozen (%)	Un-blanched frozen (%)
Mint	Water	79.58 ± 10.72	72.03 ± 9.19	93.91 ± 4.08
	Methanol	85.62 ± 5.25	84.11 ± 12.05	88.58 ± 3.18
Thyme	Water	35.32 ± 5.74	17.45 ± 2.18	18.28 ± 2.16
	Methanol	86.42 ± 5.25	80.73 ± 6.7	85.51 ± 9.5
Basil	Water	13.36 ± 1.95	40.62 ± 2.95	32.77 ± 5.71
	Methanol	87.84 ± 6.7	82.21 ± 7.13	73.82 ± 5.34

Table 4. 1 Results of the DPPH effects of different treatments of herbs. Results are means of three readings of three different experiments.

From table 4.1, the results of the DPPH inhibition ability varied across herbs, treatments and extraction solvent. For mint, ANOVA of results of water extracts showed a significant difference (P = 1.42E-12) between results of fresh, and frozen (un-blanched and blanched) samples. A further post hoc t-test of the results showed that there was a significant difference (P = 4.44E-07) between the water extracts of fresh and blanched frozen mint. However, the DPPH inhibition ability of un-blanched frozen mint was significantly higher than that of water extracts of both fresh (P = 6.63E-08) and blanched frozen (P = 2.17E-09) mint. In contrast to results of water extracts, an ANOVA of DPPH of methanol extracts showed a slight significant difference between results of all samples (P = 0.03), however, there was no significant difference (P = 0.75) between the DPPH inhibition ability of methanol extracts of blanched and un-blanched frozen mint.

Results of extracts of thyme showed a significant (P = 8.54E-07) difference between fresh and frozen samples (blanched and un-blanched). A further post hoc test showed the DPPH inhibition ability of water extracts of fresh thyme was significantly higher than that of both blanched (P = 2.54E-06) and un-blanched (P = 3.48E-04) frozen thyme. However, there is slight significant difference (P = 0.02) between the water extracts of blanched and un-blanched frozen thyme. The results of methanol extracts remained insignificant (P =0.33) between fresh, blanched and un-blanched frozen thyme.

The ANOVA of results of the DPPH ability of water extracts of basil showed a significant difference (P = 1.57E-09) between fresh, and frozen (blanched and un-blanched) samples. Furthermore, a post hoc t-test showed that blanched frozen extracts had a significantly (P = 1.46E-08) higher value than water extracts of fresh basil but not significantly different (P = 0.19) from results obtained with water extracts of un-blanched frozen basil. However, results obtained from methanol extracts of basil remain insignificant (P = 0.75) between fresh, blanched and un-blanched frozen herb.

(a)

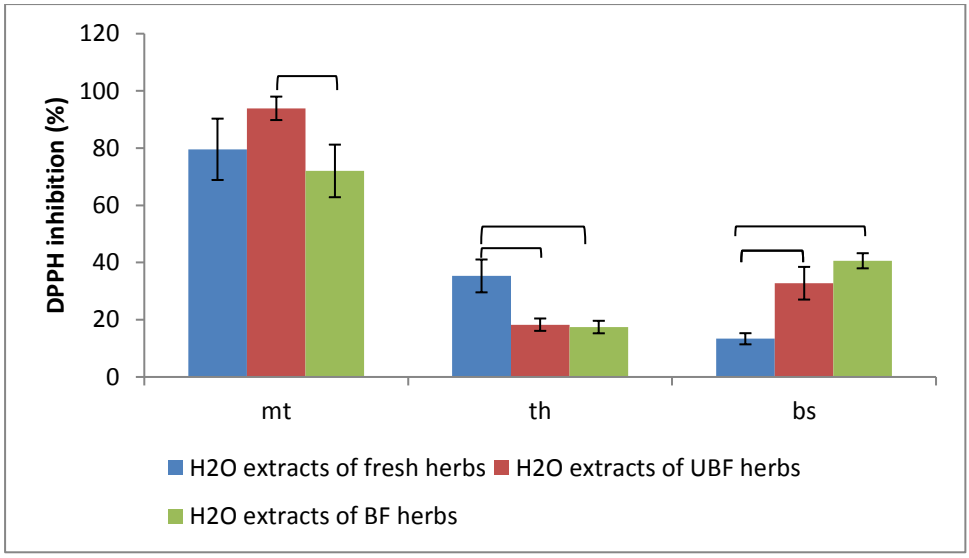


Figure 4.1 A plot of the representation of the effects of blanching and freezing on the DPPH inhibition (%) of water extracts of herbs; Mint (mt); Thyme (th) and Basil (bs). Results are means of three readings of three different experiments. Values linked with a ⊓ are significantly different (P < 0.5)

4.2.2 RESULTS OF FRAP ASSAY

Transition metals such as Fe III and Cu II are known to catalyze the initiation of radical chain reaction in lipid peroxidation. However, the presence of chelating agents may inhibit lipid oxidation by reducing these metals to more stable un-reactive compounds (Fe II and Cu I), hence the determination of the chelating ability/potential of selected herbs. The antioxidant compounds are responsible for chelating these metals. In FRAP, antioxidant compounds reduce ferric (Fe III) to ferrous (Fe II). The reduction ability is determined by measuring the coloured complex at 593nm. For this thesis, the FRAP values of different herb treatments (fresh, un-blanched and blanched frozen) extracted in different solvents (water, methanol, RMCD and 74mM PBS pH7.4) were determined and calculated as µmol Fe (II) Equivalent/L of extract using a standard curve obtained from $FeSO_4.7H_2O$.

The results of the FRAP assays summarised in Table 4.2 showed inconsistent variation between herbs, treatments and extraction solvents.

Herbs	Extraction solvent	Treatment (± standard deviation)		
		Fresh (μM Fe II equival/L extract)	Blanched frozen (μM Fe II equival/L extract)	Un-blanched frozen (μM Fe II equival/L extract)
Mint	Water Methanol	672.69 ± 66.32 3828.10 ± 304.16	1312.1 ± 32.41 1978.13 ± 10.01	1413.10 ± 42.23 2392.06 ± 18.76
Thyme	Water Methanol	348.65 ± 40.79 3373.35 ± 31.93	216.01 ± 12.02 1583.31 ± 52.13	240.96 ± 3.24 1924.83 ± 21.13
Basil	Water Methanol	149.55 ± 31.87 2541.39 ± 30.71	311.72 ± 10.21 1132.03 ± 21.09	336.21 ± 21.01 1431.53 ± 35.12

Table 4. 2 Results of FRAP of different treatments of herbs. Results are means of three readings of three different experiments.

4.2.2.1. EFFECTS OF FREEZING AND BLANCHING ON THE FRAP OF HERBS

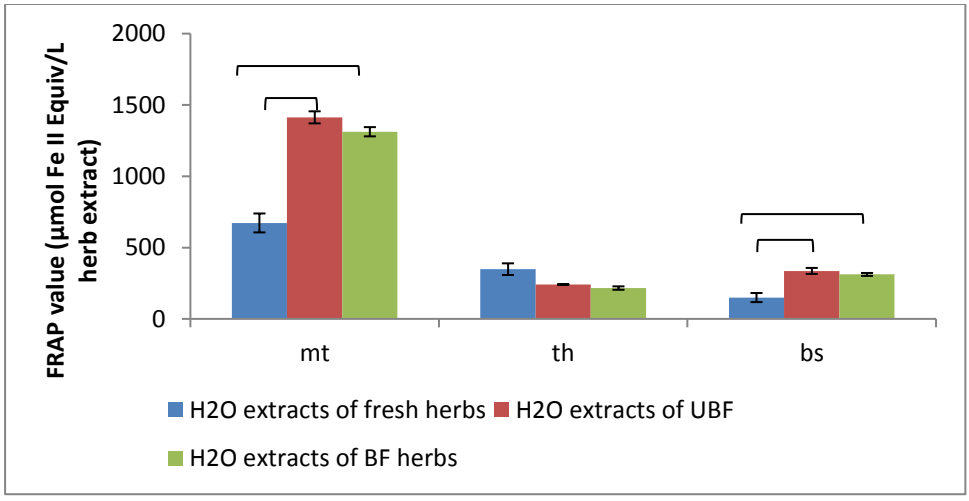
The ANOVA of results of the FRAP value of water extracts of mint showed a significant difference (P = 2.66E-12) between fresh, and frozen (un-blanched and blanched) samples. Furthermore, a post hoc t-test showed that the FRAP of water extracts of un-blanched frozen mint was significantly higher than that of water extract of fresh and blanched frozen mint (P = 9.57E-12, and P = 2.97E-03 respectively). In contrast, results of methanol extracts of fresh mint showed significantly higher FRAP values than that of both blanched and un-blanched frozen mint (P = 6.71E-09, and P = 7.87E-08 respectively). Furthermore, blanched frozen samples showed to have a significantly (1.56E-06) lower FRAP value than un-blanched frozen samples.

An ANOVA of results of FRAP of water extracts of thyme showed a significant (P = 1.17E-07) difference between fresh and frozen samples. A post hoc test showed that water extracts of fresh thyme had a significantly higher values than that of both blanched and un-blanched frozen thyme (P = 6.92E-06, and P = 4.85E-05 respectively). Furthermore, water extracts of blanched frozen samples showed to have a significantly (P = 8.27E-04) lower FRAP value tha un-blanched frozen samples. The results of the methanol extracts of thyme equally showed

significant (P = 2.81E-24) difference between FRAP values of fresh and frozen samples. However, higher FRAP values between extracts of fresh thyme and that of both blanched and un-blanched frozen thyme, however with higher level of significance (P = 4.09E-17, and P = 2.4E-16, respectively). The FRAP value of methanol extract of un-blanched frozen thyme was also significantly (P = 4.73E-12) higher than that of blanched frozen thyme.

The ANOVA of results of the water extracts of basil showed a significant difference (P = 8.31E-11) between fresh, and frozen (un-blanched and blanched) samples. Furthermore, post hoc t-test of results showed that the highest FRAP value with un-blanched frozen. FRAP value of water extracts of un-blanched frozen was significantly higher than that of fresh basil (P = 3.97E-08) but slightly significantly higher (P = 0.01) than blanched frozen basil. FRAP value of water extracts of blanched frozen basil was also significantly (P = 6.24E-08) higher than that of the fresh basil. Furthermore, ANOVA of methanol extracts of basil showed that there is a significant diffeence (P = 0.0001) between fresh, and frozen (un-blanched and blanched) samples. The highest FRAP value was obtained with extracts of fresh basil which was also significantly higher than those of both blanched (P =2.13E-04) and un-blanched (P = 1.45E-11) frozen basil. Furthermore, the FRAP value of un-blanched frozen basil was significantly higher (P = 0.0015) than that of blanched frozen basil.

(a)



(b)

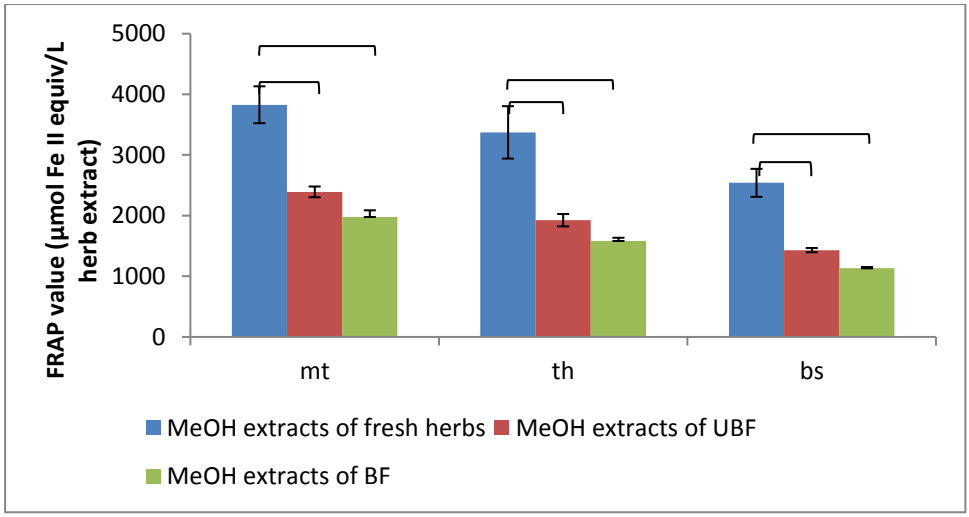


Figure 4.2 A plot of the representation of the effects of blanching and freezing on FRAP value of (a) Water; (b) Methanol extracts of fresh, un-blanched and blanched frozen herbs. Mint (mt); Thyme (th) and Basil (bs). Results are means of three readings of three different experiments. Values linked with a ⊓ are significantly different (P < 0.05)

4.2.3 RESULTS OF CUPRAC OF HERBS

The CUPRAC method of antioxidant measurement is based on the absorbance measurement of Cu (I)-neocuproine (Nc) chelate formed as a result of the redox reaction of chain-breaking antioxidants with the CUPRIC reagent, Cu(II)-Nc, where absorbance is recorded at the maximal light-absorption wavelength of 450nm. The chromogenic redox reagent used for the CUPRAC assay is bis(neocuproine) copper (II) chelate. This reagent is useful at pH 7 and the absorbance of the coloured Cu (I)-chelate formed is as a result of redox reaction with reducing polyphenols which is measured at 450 nm. In this assay, CUPRAC values of blanching and frozen herbs varied with extraction solvent and treatment given to herbs. The CUPRAC values were calculated from the standard curve of Copper 1 and expressed as mg trolox equivalent (TE)/g dw herb.

The results of CUPRAC of herbs are summarised and represented in Table and Figure 4.3 respectively.

Herbs	Extraction solvent	Treatment		
		Fresh (mg TE/g dw herb)	Blanched frozen (mg TE/g dw herb)	Un-blanched frozen (mg TE/g dw herb)
Mint	Water Methanol	54.84 ± 9.24 273.97 ± 23.77	22.02 ± 1.15 151.18 ± 5.04	7.11 ± 2.20 155.74 ± 8.19
Thyme	Water Methanol	9.14 ± 0.98 167.78 ± 36.05	12.85 ± 1.39 133.51 ± 9.18	12.88 ± 1.15 154.03 ± 2.99
Basil	Water Methanol	20.78 ± 3.07 343.09 ± 31.05	33.72 ± 2.14 181.10 ± 5.21	15.52 ± 2.02 171.00 ± 8.71

Table 4. 3 Results of CUPRAC of different treatments of herbs. Results are means of three readings of three different experiments (± standard deviation).

4.2.3.1 EFFECTS OF FREEZING AND BLANCHING ON THE CUPRAC OF HERBS

Figures 4.3 a, and b show the representation of the effect of thermal treatment (blanching) and freezing on the CUPRAC value of mint, thyme and basil. The results varied inconsistently across different treatments and extraction solvents. However, methanol extracts of all herbs had the highest CUPRAC values across all treatments.

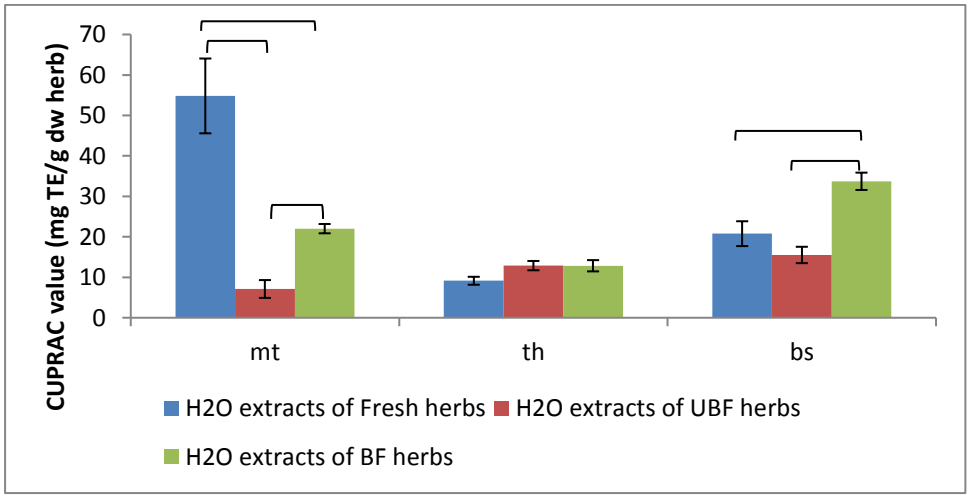
The ANOVA of CUPRAC value for water extracts of mint showed a significant difference (P = 1.98E-09) between results of fresh, and frozen samples (un-blanched and blanched). A further post hoc t-test showed that extracts of fresh mint had the highest CUPRAC value which was significantly higher than that of blanched (P = 3.04E-06) and un-blanched (P = 5.31E08) frozen mint. Furthermore, extracts of blanched frozen mint had a significantly higher (P = 0.01) CUPRAC value than that of un-blanched frozen mint.

ANOVA of results of the CUPRAC values of methanol extracts of mint showed a significant (P = 1.05E-10) difference between all samples. Furthermore, a post hoc t-test equally showed that the highest CUPRAC value was obtained with extracts of fresh mint which was significantly higher than that of both blanched (P = 1.09E-07) and un-blanched (3.63E-10) frozen mint. However, there is no significant difference (P = 0.27) between results of un-blanched and blanched frozen samples.

The ANOVA of results obtained with water extracts of thyme showed a significant (P = 4.9E-04) difference between fresh, and frozen (un-blanched and blanched frozen) samples. A further post hoc t-test showed that frozen herbs (blanched and un-blanched) had a significantly higher (P = 5.58E-03, and P = 6.23E-04, respectively) CUPRAC values than that of fresh thyme. However, there is no significant (P = 0.06) difference between blanched and un-blanched frozen samples. ANOVA of results of CUPARC values of methanol extracts showed a significant (P = 8.39E-04) difference between fresh and frozen samples. Furthermore, extracts of fresh thyme significantly had the highest CUPRAC value compared to those of blanched (P = 4.42E-03) but not significantly (P = 0.99) different from un-blanched frozen thyme. Furthermore, extracts of blanched frozen samples showed the least CUPRAC value which is significantly (P = 1.76E-05) lower than un-blanched frozen samples.

The ANOVA of CUPRAC values obtained with water extracts of basil showed that there was a significant difference (P = 7.9E-08) between all samples. Furthermore, blanched frozen basil showed to have the highest CUPRAC value which is significantly higher than both fresh (P = 1.48E-08) and un-blanched (P = 1.17E-08) frozen basil. There was no significant difference (P = 0.84) between water extracts of fresh and un-blanched frozen basil. An ANOVA of results of methanol extracts of basil samples showed a significant difference (P = 1.37E-12) between fresh and frozen samples. Furthermore, results showed that of all the treatments, extracts from fresh basil had the highest CUPRAC value, which is significantly different from that of both blanched (P = 1.19E-08) and un-blanched (P = 5.73E-09) frozen basil. However, there was no significant difference (P = 0.19) between un-blanched and blanched frozen samples.

(a)



(b)

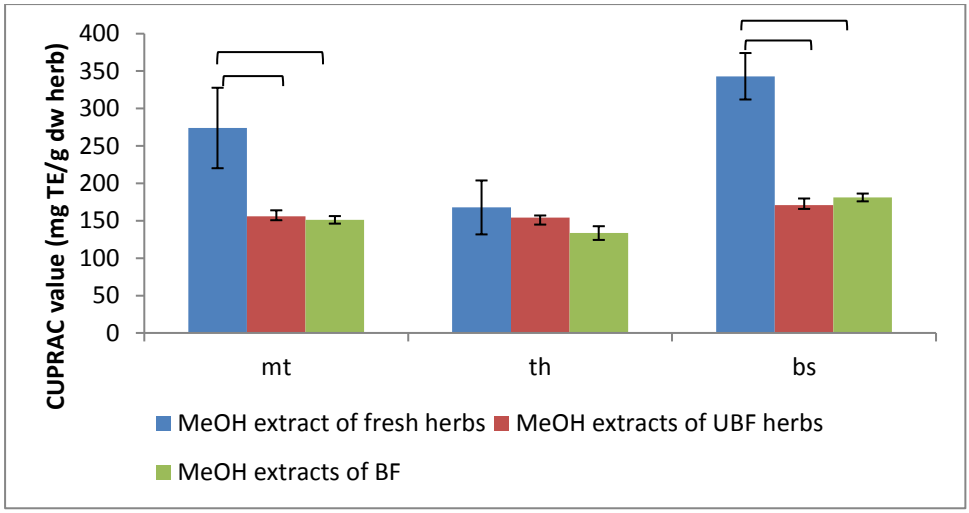


Figure 4.3 Microsoft Excel plot showing the effects of blanching and freezing on the CUPRAC value of (a) Water; (b) Methanol extracts of fresh, un-blanched and blanched frozen herbs. Mint (mt); Thyme (th) and Basil (bs). Results are means of three readings of three different experiments. Values linked with a ⊓ are significantly different ($P < 0.05$)

4.2.4 RESULTS OF ORAC OF HERBS

The ORAC assay investigated the protection afforded by the antioxidant compound (from selected herbs) to a target molecule (fluorescein) that is being oxidized by peroxyl radicals (AAPH), estimating the changes of area under the fluorescein decay curve and the kinetics profile of loss in fluorescence. Results of ORAC values of herbs are summarised in Table 4.4

Herbs	Extraction solvent	Treatment (± standard deviation)		
		Fresh (mg TE/g dw herb)	Blanched frozen (mg TE/g dw herb)	Un-blanched frozen (mg TE/g dw herb)
Mint	Water	44.12 ± 9.02	129.77 ± 3.17	161.05 ± 3.61
	Methanol	111.28 ± 29.49	120.16 ± 6.72	94.80 ± 7.53
Thyme	Water	40.71 ± 3.62	28.10 ± 1.05	32.10 ± 5.21
	Methanol	56.28 ± 8.24	60.16 ± 3.02	61.32 ± 2.35
Basil	Water	75.11 ± 10.57	30.16 ± 3.91	35.41 ± 6.10
	Methanol	81.43 ± 12.56	59.92 ± 4.21	72.48 ± 10.04

Table 4. 4 Results of ORAC of different treatments of herbs. Results are means of three readings of three different experiments.

4.2.4.1 EFFECTS OF FREEZING AND BLANCHING PRIOR TO FREEZING ON THE ORAC VALUES OF HERBS

Figure 4.4 shows the representation of the effects of blanching and freezing on the ORAC value of herbs with an inconsistent variation among treatments and crude extracts. The ANOVA of ORAC values of water extracts of mint showed a significant difference (P =3.36E-16) between all samples. Furthermore, extracts of un-blanched frozen mint showed to have a significantly higher ORAC value than that of both fresh (P = 1.96E-12), and blanched (P = 9.4E-08) frozen mint. Result of methanol extracts of mint showed that there is a significant (P = 1.19E-03) difference between fresh and frozen (blanched and un-blanched) samples. Furthermore, the highest ORAC value was obtained with methanol extracts of blanched frozen mint which was significantly different to the ORAC value of extracts of fresh mint (P = 0.03), and un-blanched frozen (P = 1.89E-04) mint.

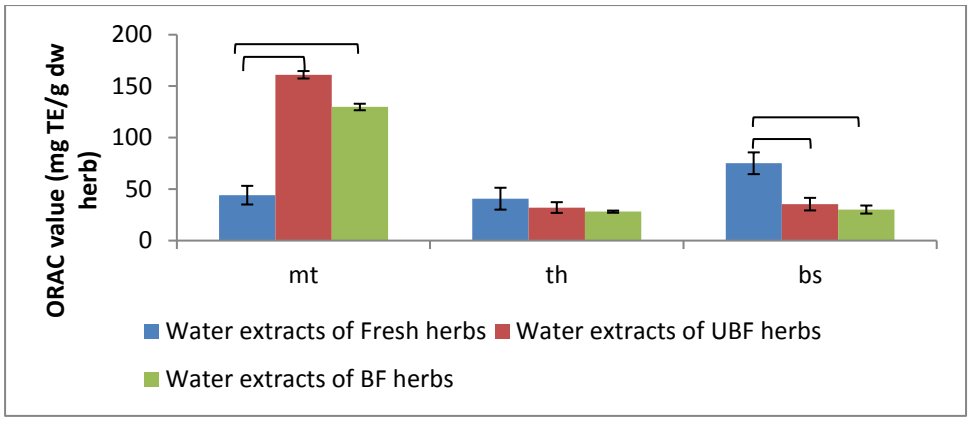
Generally, among all extracts from thyme, methanol extracts of un-blanched frozen thyme had the highest ORAC value. However, the ANOVA of water extracts showed a significant difference (P = 3.92E-05) between all herb samples. Furthermore, a post hoc t-test showed that fresh thyme had the highest ORAC value which is not significantly (P = 0.07) higher than un-blanched frozen samples, but significantly (P = 2.50E-06) higher than the ORAC value of blanched frozen sample. Blanched frozen samples were shown to have an ORAC value significantly different (P = 3.46E-03) to that of un-blanched frozen samples.

Results of ORAC of methanol extracts of thyme showed that there is no significant difference (P = 0.075) between fresh and frozen (blanched and un-blanched) samples. However, un-blanched frozen samples showed to have the highest ORAC value while the fresh samples showed the least ORAC value.

The ANOVA of ORAC values water extracts of basil showed a significant difference (P = 2.9E-09) among all the samples. However, the significantly highest ORAC value was obtained with fresh sample compared to blanched (P = 1.55E-07) and un-blanched (P = 1.09E-06) frozen basil. The least was obtained with blanched frozen samples which is also significantly (P = 1.59E-03) lower than the ORAC value of un-blanched frozen samples. For methanol extracts, there is a significant (P = 4.11E-06) difference between the ORAC values of all samples. Furthermore, the highest ORAC value was obtained with the methanol extracts of fresh basil followed by un-blanched frozen basil. Methanol extracts of blanched frozen basil possessed a

significantly lower ORAC value compared to fresh (P = 5.57E-06), and un-blanched frozen (P = 1.2E-04) basil.

(a)



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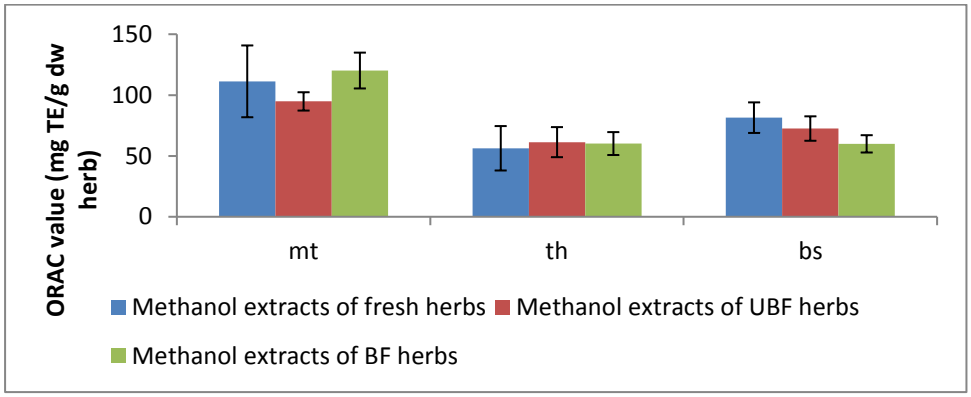
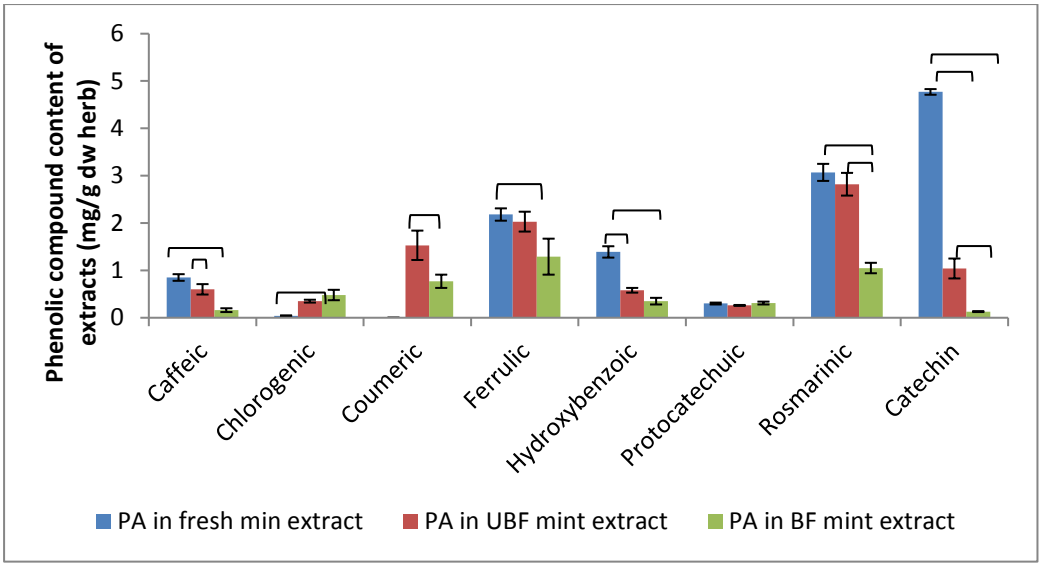


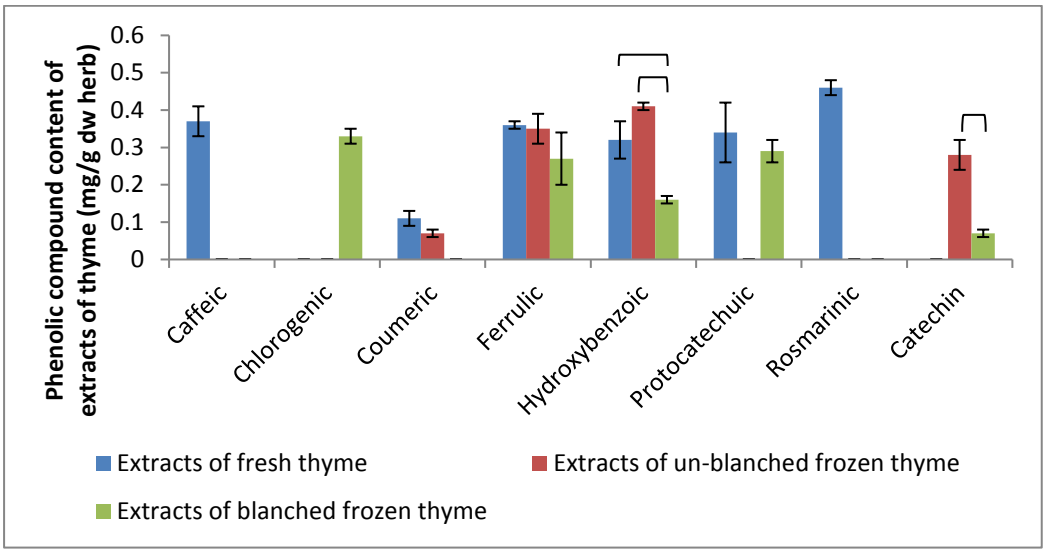
Figure 4.4 Microsoft Excel plot of the representation of the effects of blanching and freezing on ORAC value of (a) Water and (b) Methanol extracts of fresh, un-blanched and blanched frozen herbs. Mint (mt); Thyme (th) and Basil (bs). Results are means of three readings of three different experiments. Values linked with a ∩ are significantly different (P < 0.05)

4.3 RESULTS OF HPLC ANALYSIS OF PHENOLIC COMPOUND PROFILE OF AQUEOUS EXTRACTS OF HERBS

Analysed individual phenolic acids were chosen based on previous reports on the phenolic compound content of *Lamaiceae* herbs and their enzyme inhibition effectiveness (Cheplick, *et al.*, 2010; Kwon, *et al.*, 2006; Wongsa, *et al*, 2012).


Phenolic compound content of extracts (mg/g dw herb)
6
5
4
3
2
1
0
Caffeic
Chlorogenic
Coumeric
Ferrulic
Hydroxybenzoic
Protocatechuic
Rosmarinic
Catechin
PA in fresh min extract
PA in UBF mint extract
PA in BF mint extract

(a)


Phenolic compound content of extracts of thyme (mg/g dw herb)
0.6
0.5
0.4
0.3
0.2
0.1
0
Caffeic
Chlorogenic
Coumeric
Ferrulic
Hydroxybenzoic
Protocatechuic
Rosmarinic
Catechin
Extracts of fresh thyme
Extracts of un-blanched frozen thyme
Extracts of blanched frozen thyme

(b)

(c)

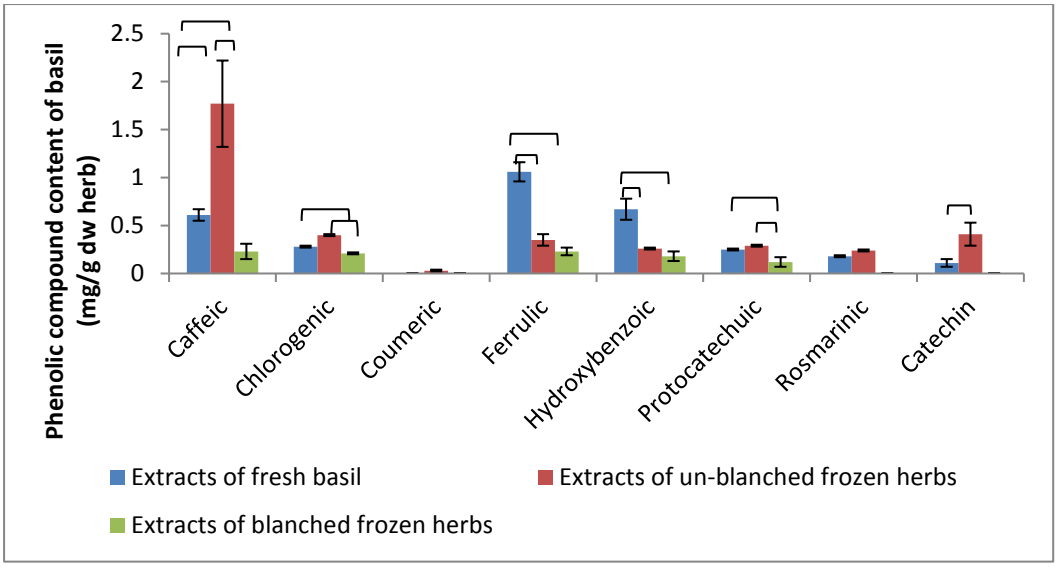


Figure 4.5 Microsoft Excel plot showing a representation of the HPLC determination of individual phenolic compounds of fresh, un-blanched frozen (UBF) and blanched frozen (Bf) (a) mint, (b) thyme and (c) basil extracts. Results are means of three readings of three different experiments. Values linked with ∩ are significantly different (P<0.05)

Figure 4.5 a, b and c shows results of individual phenolic compounds of water extracts of herbs. From the results, mint possesses the highest phenolic compound content.

With mint extracts, the results show that individual phenolic compounds varied among treatments with fresh mint generally having the highest values across most assayed phenolic compounds. In fresh mint extract, the phenolic compound contents are in the order catechin > rosmarinic acid >ferulic acid > hydroxybenzoic acid > caffeic acid > protocatechuic acid > chlorogenic acid. Furthermore, fresh mint sample showed to have the highest catechine, caffeic, ferulic and rosmarinic acid content while un-blanched frozen samples had the least.

An ANOVA of results of catechin content of mint samples showed that there is a significant difference (P = 2.74E-12) between fresh and frozen (un-blanched and blanched) samples. A further post hoc t-test showed that there is a significant differenc between the catechine content of fresh compared to un-blanched (P = 3.55E-07), and blanched (P = 2.13E-09) frozen samples. There is also a significant difference (P = 1.63E-04) between catechin content of un-blanched and blanched frozen.

For results of chlorogenic acid content of mint, fresh samples showed to have the least value while the blanched frozen samples had the highest value. The ANOVA of result values showed

a significant difference (P = 1.13E-04) between all samples, however, a post hoc t-test showed no significant difference (P = 0.11) between blanched and un-blanched frozen samples.
Results of the coumaric acid content of mint showed that no coumeric acid was detected in fresh extracts, however, un-blanched frozen samples showed significantly (P = 6.69E-05) higher value compared to blanched frozen samples. For the ferulic acid content of mint, fresh samples showed the highest value while blanched frozen samples had the least value. ANOVA of result data showed a significant difference (P = 0.001) between all samples, however, there was no significant difference (P = 0.69) between fresh and un-blanched frozen samples. Furthermore, blanched frozen showed to be significantly lower than both fresh (P = 1.05E-03) and un-blanched frozen (P = 0.02) samples.
The results of the protocatecheuic acid content of mint showed a no significant difference between all samples. An ANOVA of result data of rosemarinic acid showed a significant difference (P = 2.81E-04) between fresh and frozen (un-blanched, and blanched) samples. A further post hoc t-test showed a no significant (P = 0.48) difference between the rosemarinic acid content of fresh, and un-blanched frozen samples.

Results of phenolic acid content of thyme showed that caffeic and rosemarinic acids were only detected in fresh samples, while chlorogenic acid was detected only in blanched frozen samples. Furthermore, coumaric acid was only detected in fresh, and un-blanched frozen samples. Results showed that fresh thyme sample had a significantly (P = 0.015) higher coumaric acid content than un-blanched frozen samples. However, catechin was only detected in un-blanched and blanched frozen thyme, with un-blanched frozen sample showing a significantly higher (P = 2.84E-09) content that blanched frozen. Protocatcheuic acid was only detected in fresh, and blanched frozen samples, with no significant difference (P = 0.59) between the two samples. Ferrulic and hydroxybenzoic acids were the only phenolic acids to be detected in all the samples of thyme. For ferulic acid, there is no significant (P = 0.06) difference between fresh, un-blanched and blanched frozen samples. A further post hoc t-test showed a significant difference (P = 0.002) between fresh samples and blanched frozen, while there is no significant difference (P = 0.88) between fresh and un-blanched frozen samples.
Results of hydroxybenzoic acid content of thyme showed that among all samples, un-blanched frozen samples had the highest value while blanched frozen showed the least value. The ANOVA of result data showed a significant (P = 1.9E-09) difference between all samples. A further post hoc t-test equally showed a significant difference between fresh and un-blanched

frozen (P = 2.05E-03), fresh and blanched frozen (P = 2.28E-05), and blanched and un-blanched frozen (P = 1.7E-07).

Results of phenolic acid content of basil showed that only slight amount (0.13mg/g dw of herb) of coumaric acid was detected in un-blanched frozen sample. Furthermore, rosemarinic acid and catechin were only detected in fresh and un-blanched frozen samples with the un-blanched frozen sample having higher rosemarinic acid and catechin content than fresh samples. Furthermore, there is no significant difference (P = 0.08) between the rosemarinic acid content of fresh, and un-blanched frozen samples. However, there was a significant difference (P = 0.004) between the catechin content of fresh, and un-blanched frozen samples.

The results of the caffeic acid content of basil showed that un-blanched frozen samples had the highest caffeic acid content while blanched frozen samples had the least. An ANOVA of result data showed a significant difference (P = 4.38E-12) between all samples. Further post hoc t-test showed a significant difference (P = 2.81E-08) between un-blanched frozen samples and fresh sample, and blanched frozen samples. Furthermore, there was also a significant (P = 3.01E-06) difference between fresh and blanched frozen. Furthermore, un-blanched frozen basil also showed the highest chlorogenic and protocatechuic acids content compared to fresh, and blanched frozen samples, while blanched frozen samples showed the least.

For the ferrulic and hydroxybenzoic acid content of basil, fresh extracts were shown to contain the highest phenolic acids with the blanched frozen containing the least. An ANOVA of result data showed a significant difference in the ferulic (P = 0.0003) and hydroxybenzoic (P = 0.002) acids between all the samples. However, for hydroxybenzoic acid, there was no significant difference (P = 0.23) between un-blanched and blanched frozen samples. Furthermore, there was a slight significant difference (P = 0.02) between un-blanched and blanched frozen samples.

4.4 COMPARISONS BETWEEN TOTAL ANTIOXIDANT AND PHENOLIC ACID CONTENT OF CRUDE HERB EXTRACTS WITH PURE/SYNTHETIC ANTIOXIDANTS

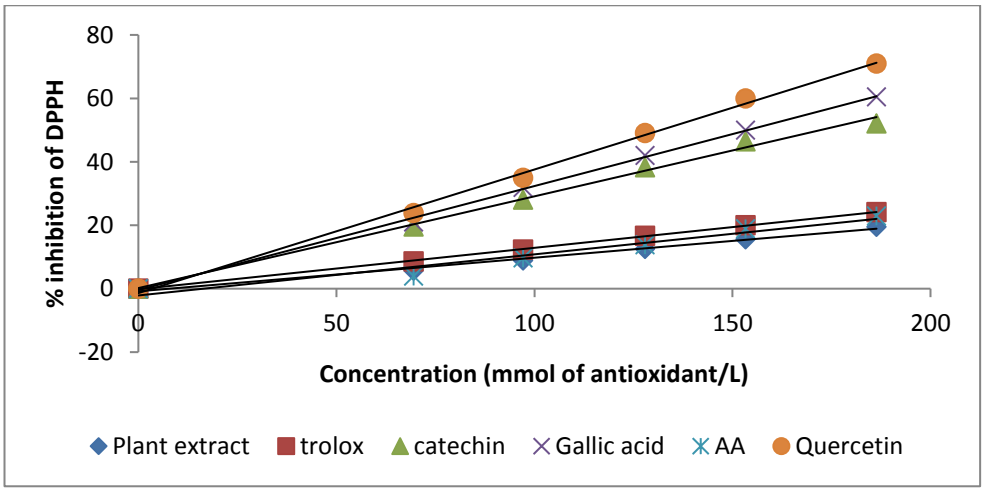
From the antioxidant tests, extracts of adequately diluted herbs (Mint, Thyme and basil) were selected for the comparison between the antioxidant activity of herbs measured as DPPH inhibition and FRAP and that of several pure antioxidant compounds (ascorbic acid, trolox, Fe^{II}, catechin, Gallic acid, Quercetin). DPPH and FRAP assays were chosen for further

antioxidant assays because of their common use in the determination of antioxidant activity of pure compounds and crude plant extracts of herbs (Addai, *et al*, 2013; Kim *et al*, 2013; Panchawat and Sisodia, 2010; Rajauria, *et al*, 2012). Furthermore, from methodological point of view DPPH and FRAP have been proposed as easy and accurate with highly reproducibility compared to other antioxidant activity assays.

4.4.1 Comparison between the antioxidant activities determined as free radical scavenging ability (DPPH) of total antioxidant content (mmol TE/L) of herb extracts measured as percentage inhibition and those of pure synthetic antioxidant compounds.

Figures 4.6 – 4.8 show the results of the free radical (DPPH) scavenging ability of different concentrations of adequately diluted crude herb extracts (prepared at room temperature) compared to similar concentrations of pure synthetic antioxidant compounds (trolox, catechin, ascorbic acid, quercetin and Gallic acid). The antioxidant concentration of crude herbs is calculated in mmol trolox equivalent (TE) per litre of herb extract (mmol TE/L). The addition of 150µl of differently treated crude herb extract (fresh, un-blanched and blanched frozen: mint, thyme and basil) to 2450µl of DPPH resulted in a rapid decline in the absorbance of DPPH at 517nm. However, decline/change in absorbance depended on antioxidant content/concentration.

(a)



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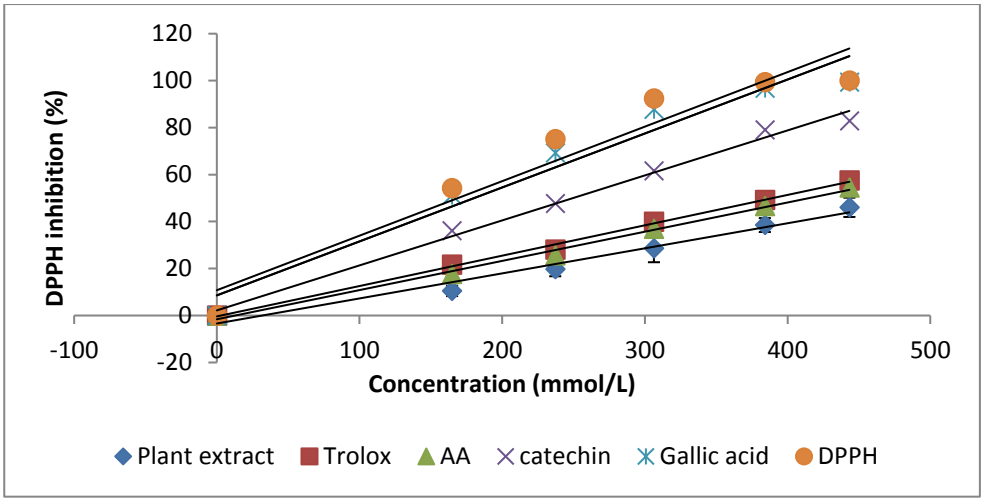

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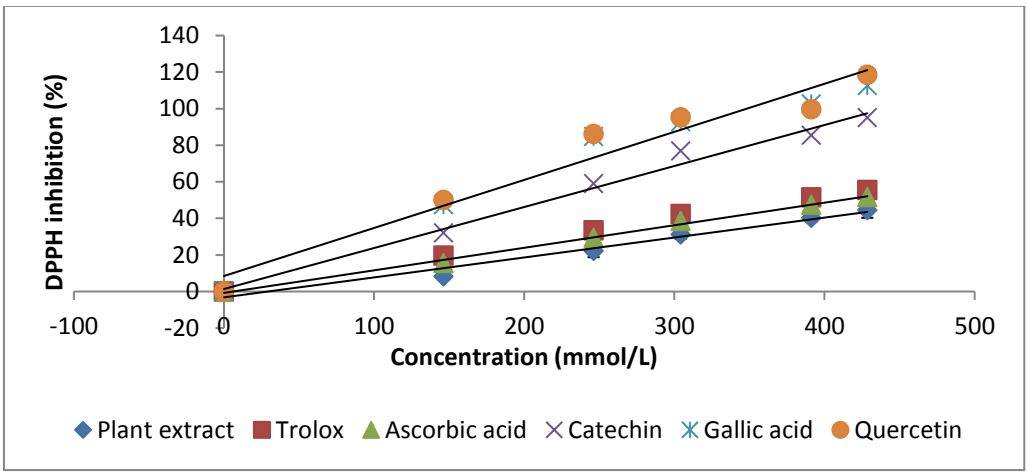
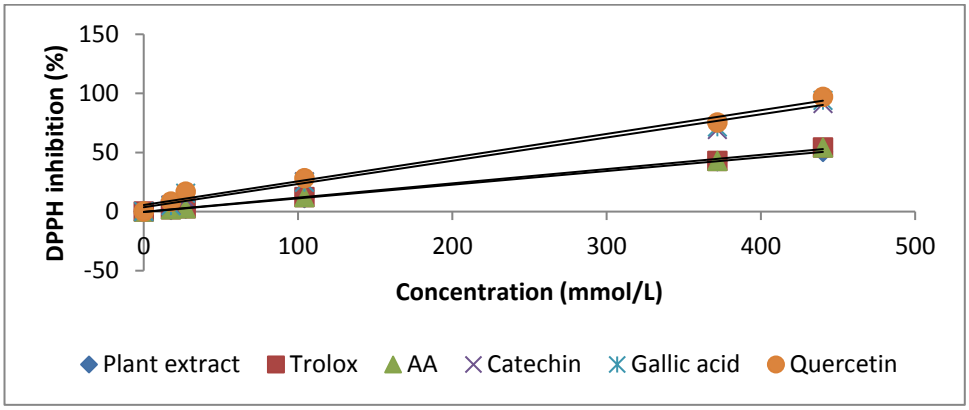


Figure 4.6 Plot of the percentage of inhibition of free radical (DPPH) in the presence of water extracts from fresh, un-blanched frozen and blanched mint (Figures 4.6 a, b and c respectively), trolox, ascorbic acid, catechin and Gallic acid over the same concentration range. Concentration is expressed as mmol of TE/L of plant extract/pure antioxidant. All results are mean of three readings.

(a)



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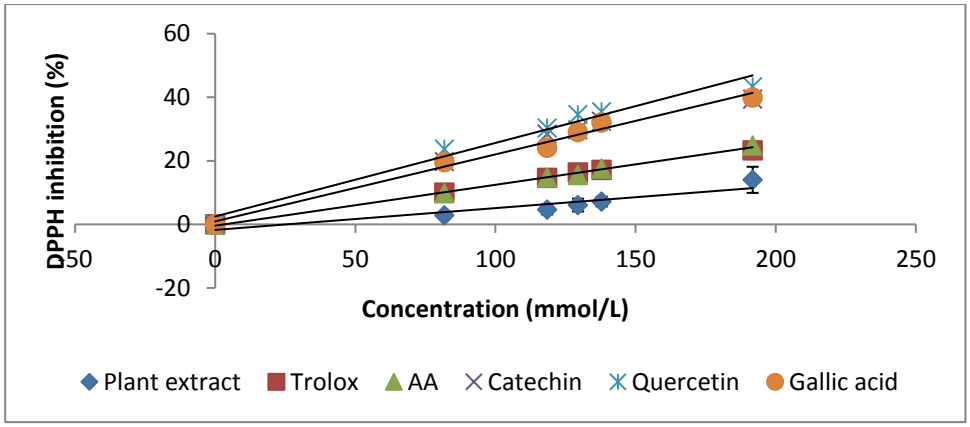

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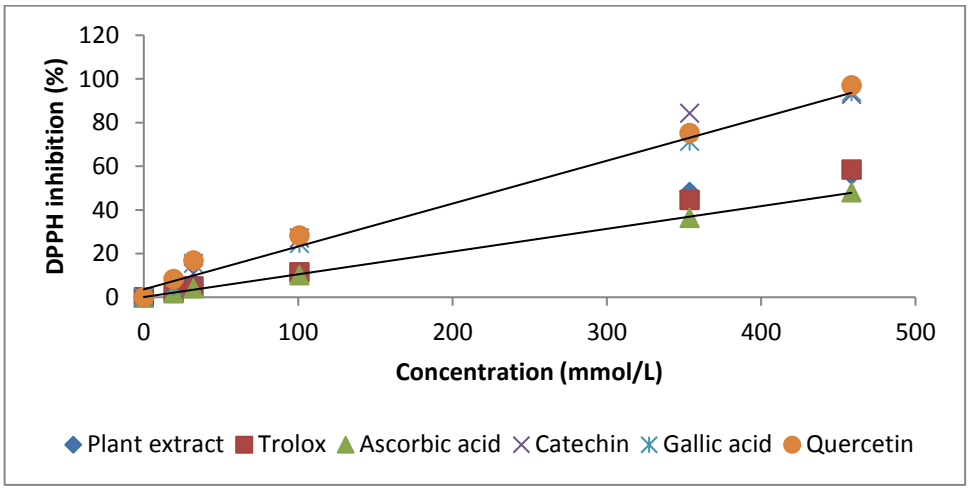
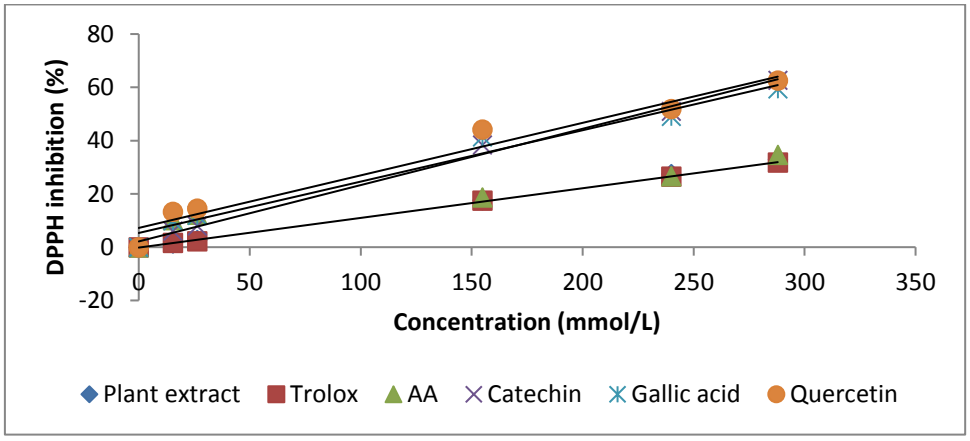
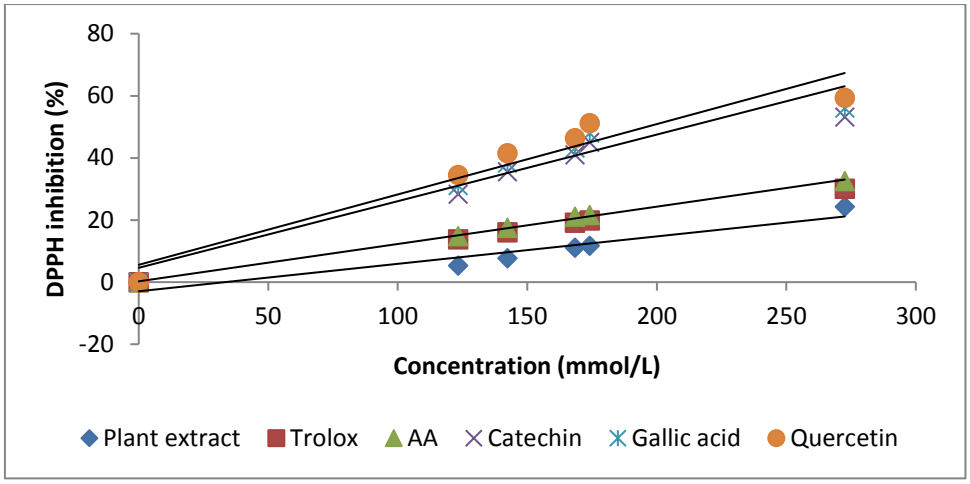


Figure 4.7 Plot of the percentage of inhibition of free radical (DPPH) in the presence of water extracts from fresh, un-blanched frozen and blanched thyme (Figures 4.7 a, b and c respectively), trolox, ascorbic acid, catechin and Gallic acid over the same concentration range. Concentration is expressed as mmol of TE/L of plant extract/pure antioxidant. All results are mean of three readings.

(a)



(b)



(c)

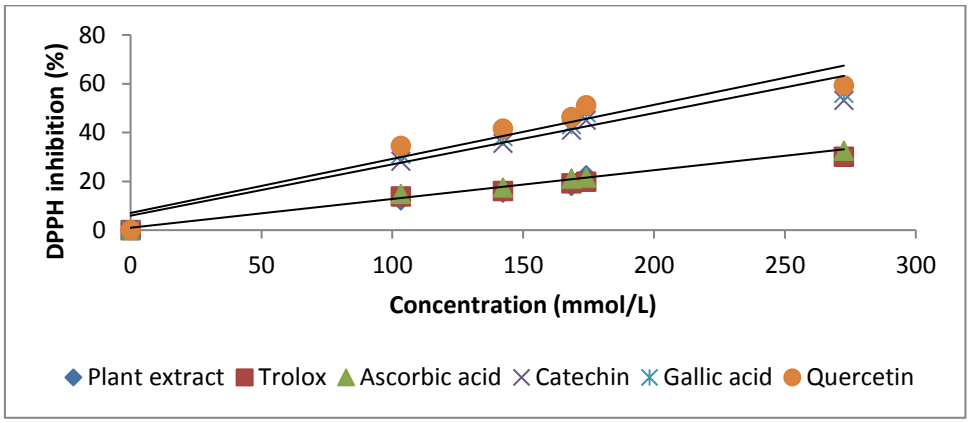


Figure 4.8 Plot of the percentage of inhibition of free radical (DPPH) in the presence of water extracts from fresh, un-blanched frozen and blanched basil (Figures 4.8 a, b and c respectively), trolox, ascorbic acid, catechin and Gallic acid over the same concentration range. Concentration is expressed as mmol of TE/L of plant extract/pure antioxidant. All results are mean of three readings.

From the results on the comparison between the DPPH of herb extracts and those of synthetic antioxidants generally shows that the antioxidant content (mmol TE/L) of crude fresh extracts of selected herbs have comparable/similar DPPH inhibition activity as trolox and ascorbic acid. Furthermore, freezing without blanching reduced the antioxidant activity measured as DPPH inhibition activity, however, blanching prior to freezing maintained the DPPH inhibition activity of the antioxidant content of selected herb extracts.

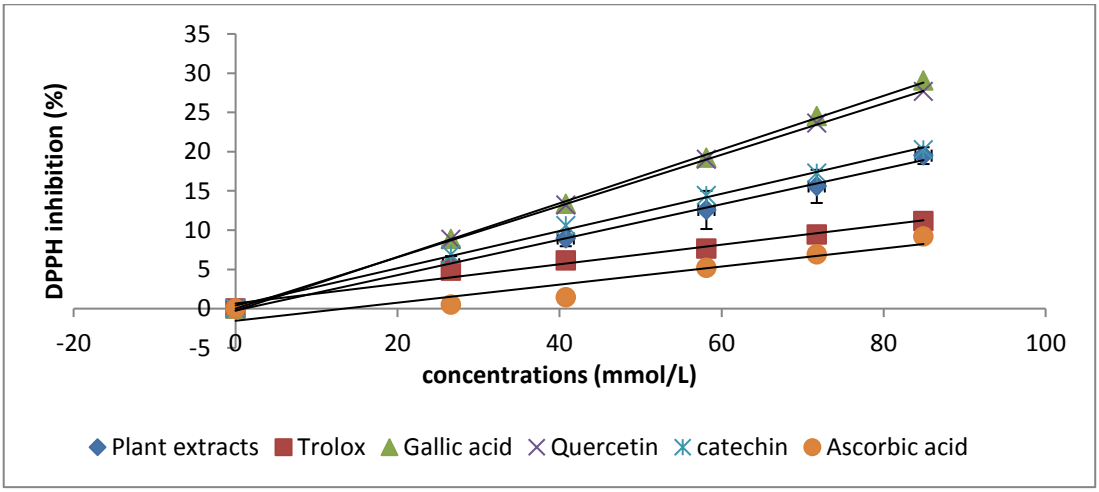
4.4.1.2 Comparison between the free radical scavenging ability (DPPH) of total phenolics content of herb extracts measured as mmol Gallic acid equivalent /L and pure phenolic and antioxidant compounds.

The free radical scavenging ability of DPPH by herb phenolic acids was determined following similar methods as those of plant total antioxidant content (mmol TE/L), however, the herb phenolic acid content was measured as mmol Gallic acid equivalent per litre of herb extract (mmol GAE/L).

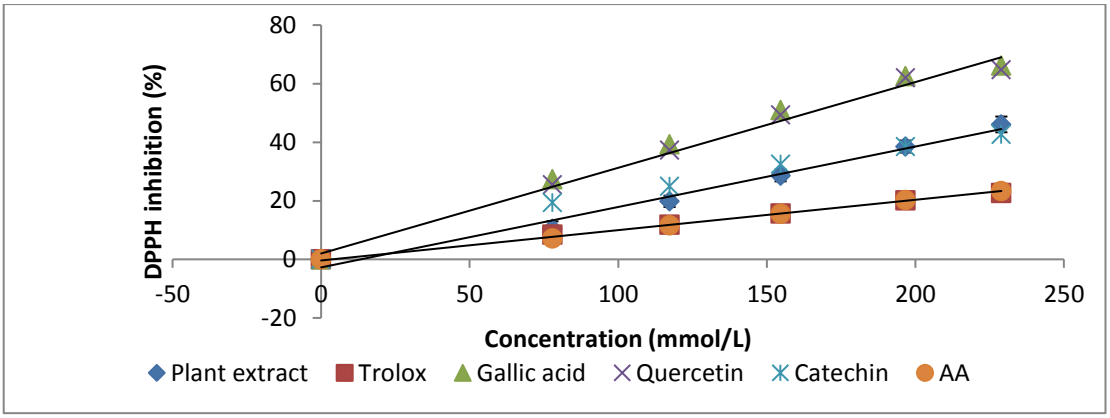
Figures 4.9 - 4.11 shows the representation of the DPPH inhibition ability of the phenolic content of crude herb extracts and pure antioxidant compounds. The DPPH inhibition of the phenolic content of mint extracts is similar/comparable to (+) catechin, significantly ($p<0.05$) better than trolox and ascorbic acid but not as good as quercetin and gallic acid across all treatments (Figures 4.9 a, b and c).

In contrast, only the extracts of fresh thyme (Figure 4.10 a) showed comparable and higher DPPH inhibition results with trolox, ascorbic acid and catechin. However, freezing (blanched and un-blanched) greatly reduced the DPPH inhibition ability of extracts were reduced and not comparable with those of assayed synthetic antioxidant compounds (Figures 4.10 b and c). Similar results were obtained with extracts of basil (Figure 4.11 a, b, and c).

(a)



(b)



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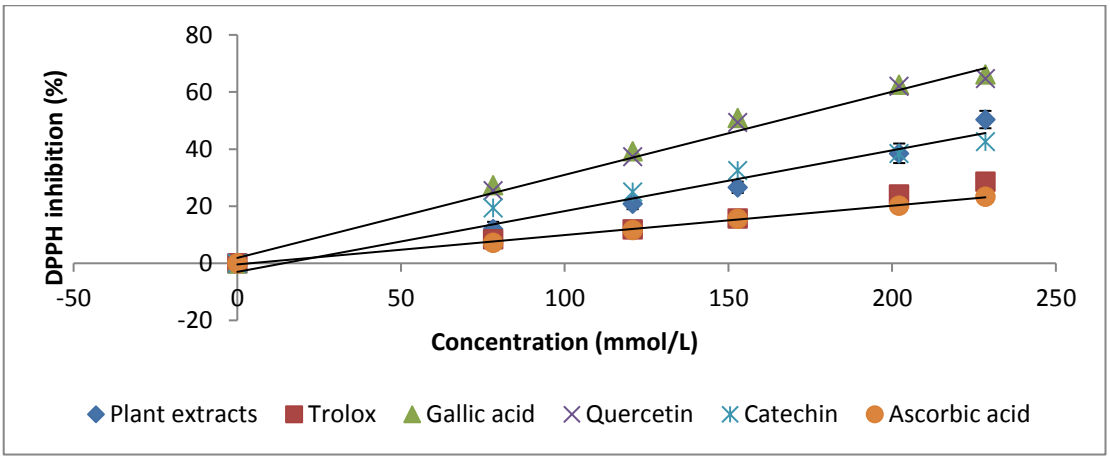
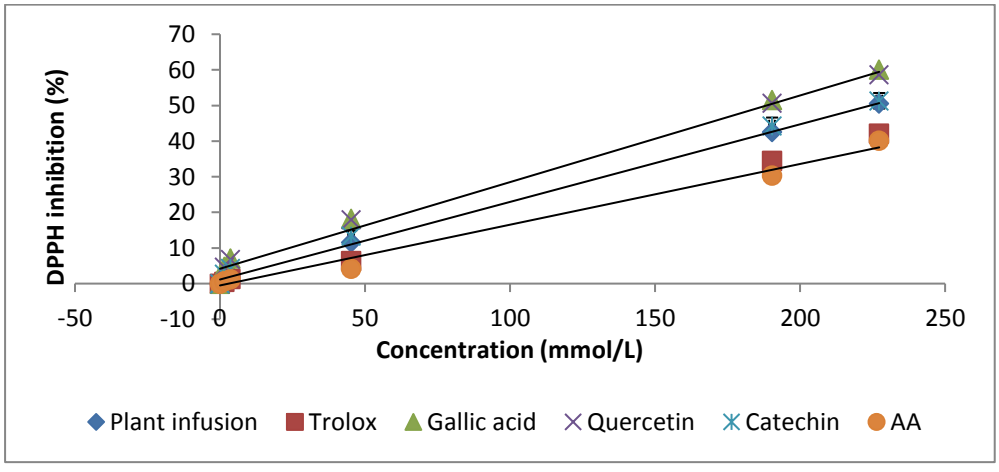


Figure 4.9 Plot of the percentage of inhibition of free radical (DPPH) in the presence of water extracts from fresh, un-blanched and blanched mint (Figures 4.9 a, b and c respectively), trolox, ascorbic acid, catechin and Gallic acid over the same concentration range. Concentration is expressed as mmol of GAE/L of plant extracts/pure antioxidant. All results are mean of three readings.

(a)



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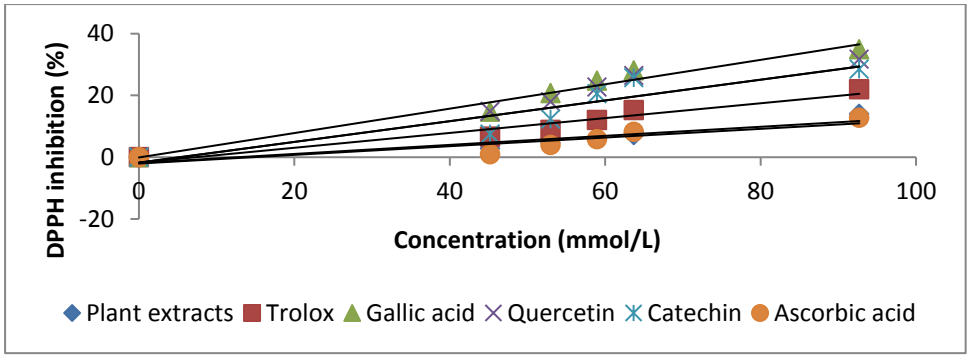


(c)

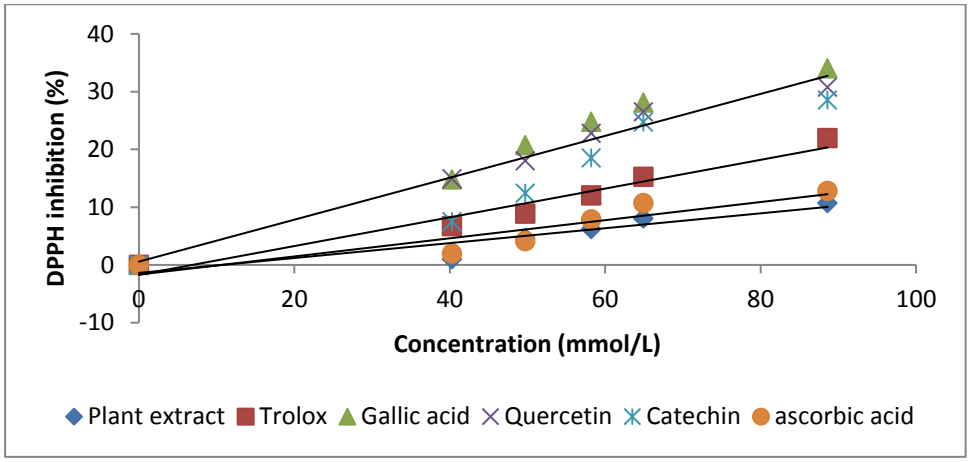
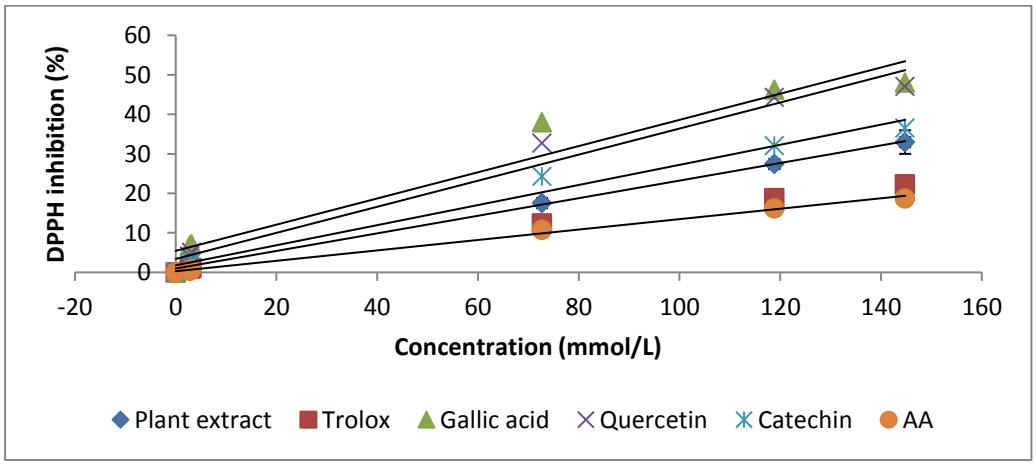
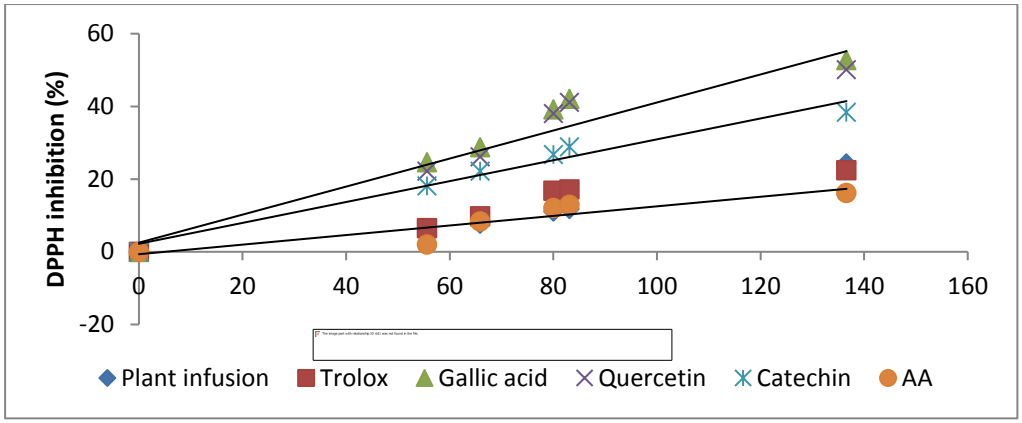


Figure 4.10 Plot of the percentage of inhibition of free radical (DPPH) in the presence of water extracts from fresh, un-blanched and blanched frozen thyme (Figures 4.10 a, b and c respectively), trolox, ascorbic acid, catechin and gallic acid over the same concentration range. Concentration is expressed as mmol of GAE/L of plant extract/pure antioxidant. All results are the mean of three readings.

(a)



(b)



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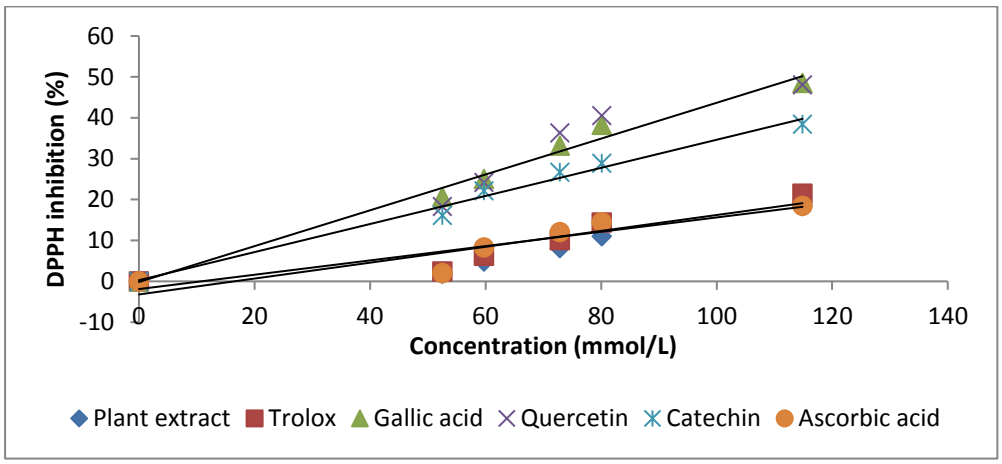


Figure 4.11 Plot of the percentage of inhibition of free radical (DPPH) in the presence of water extracts from fresh, un-blanched and blanched frozen basil (Figures 4.11 a, b and c respectively), trolox, ascorbic acid, catechin and gallic acid over the same concentration range. Concentration is expressed as mmol of GAE/L of plant extract/pure antioxidant. All results are mean of three readings.

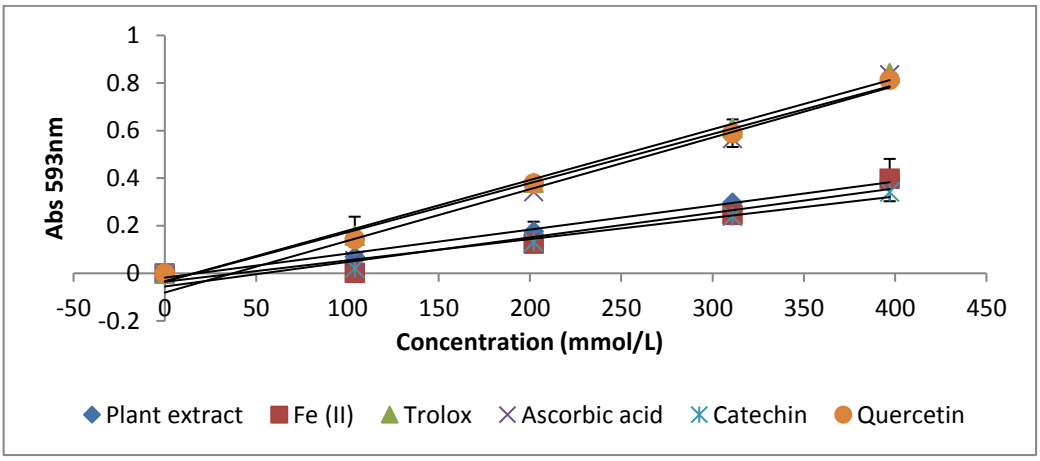
4.4.2 Comparison between the total antioxidant capacities (FRAP) of herb extracts and pure antioxidant compounds

Figures 4.12 - 4.14 shows the representation of the FRAP of different adequately diluted herb extracts and FRAP of pure antioxidant compounds. Trolox, catechin, Fe^{II}, Ascorbic acid and quercetin were determined. Total antioxidant content of herbs is measured as mmol Fe^{II} equivalent/L of herb extracts.

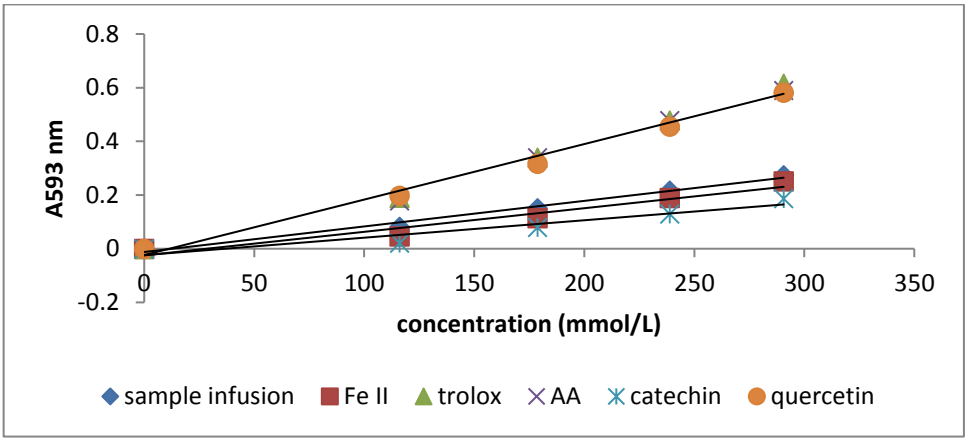
Figure 4.12 shows a dose-response characteristics of mint extracts and individual pure antioxidant compounds in the FRAP assay. Whereas the different antioxidant efficiencies is evident, the dose-response line of each individual antioxidant compounds and the FRAP of herb extracts (mmol Fe^{II}/L of mint extract) is linear.

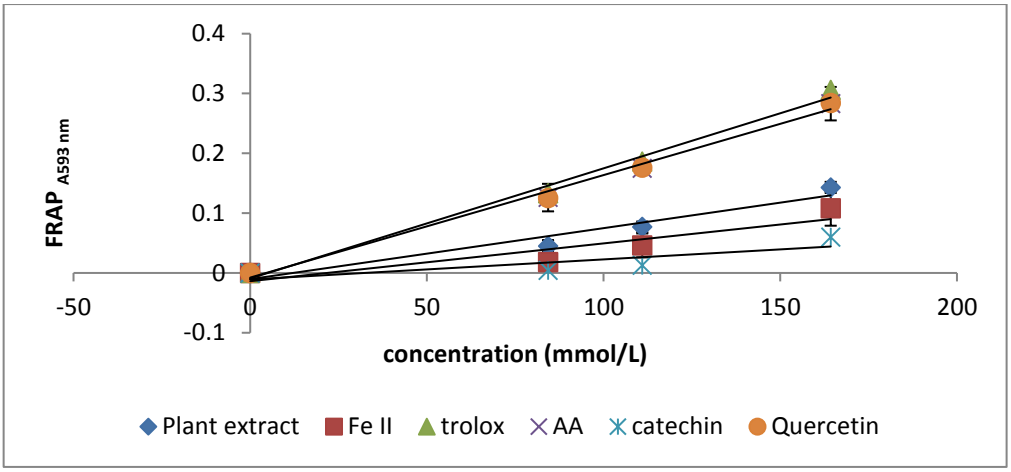
The relative antioxidant efficiency/effectiveness determined as FRAP of antioxidant content (mmol Fe^{II}/L) of fresh and un-blanched frozen herb extract (Figures 4.12a and b respectively) determined as FRAP is comparable to those of Fe II and (+) catechin but not as good as quercetin, trolox and ascorbic acid. In contrast, the FRAP of antioxidant content (mmol Fe^{II}/L) of blanched mint extracts was slightly higher than those of Fe II and (+) catechin but still not as good as quercetin, ascorbic acid and gallic acid (Figure 4.12c)

(a)



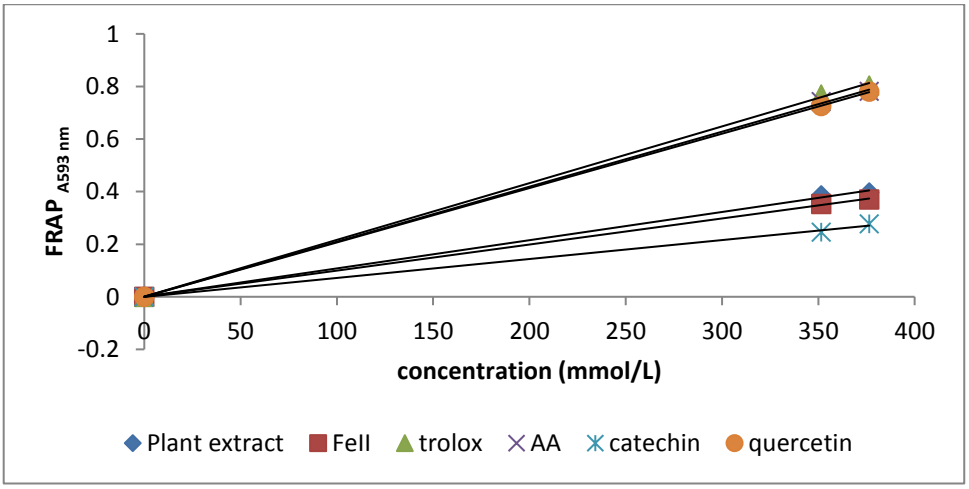
(b)



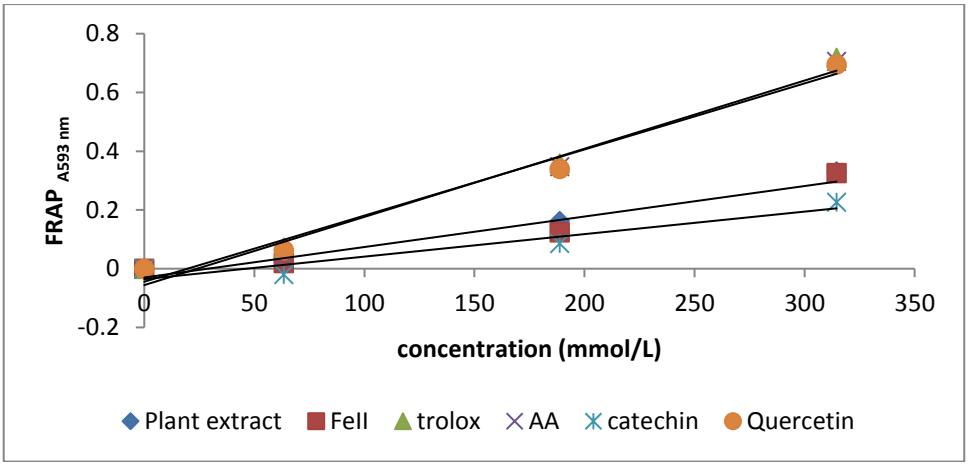


(c)

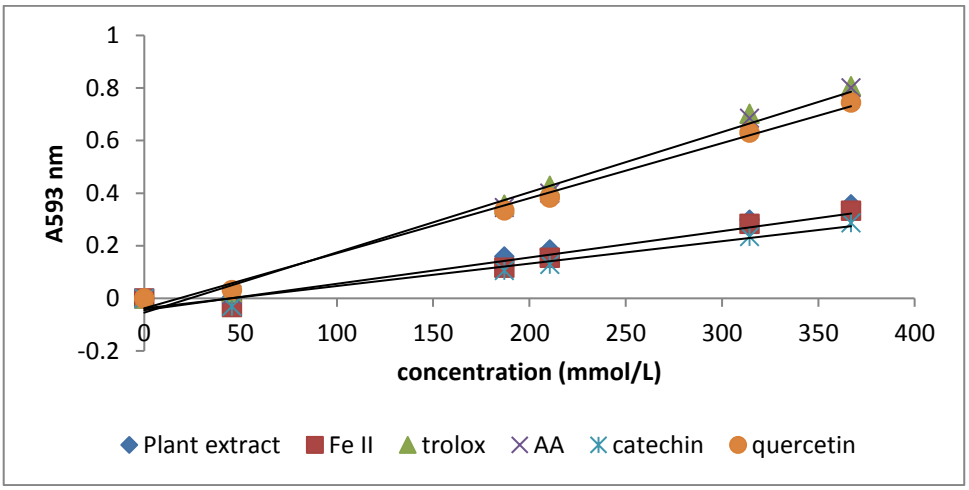
Figure 4.12 Plot of the dose- response line of the total antioxidant of water extracts of fresh, un-blanched and blanched frozen mint (4.12 a, b and c respectively), Catechin, Trolox, Ascorbic acid, quercetin and Fe^{II} over the concentration range in the ferric reducing/antioxidant power test (FRAP) assay for reducing (antioxidant) activity. Each point represents the mean of three readings



(a)

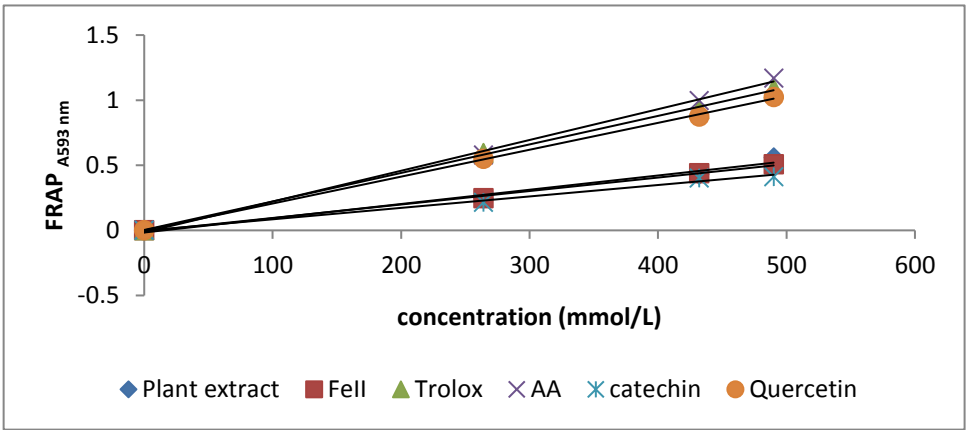


(b)



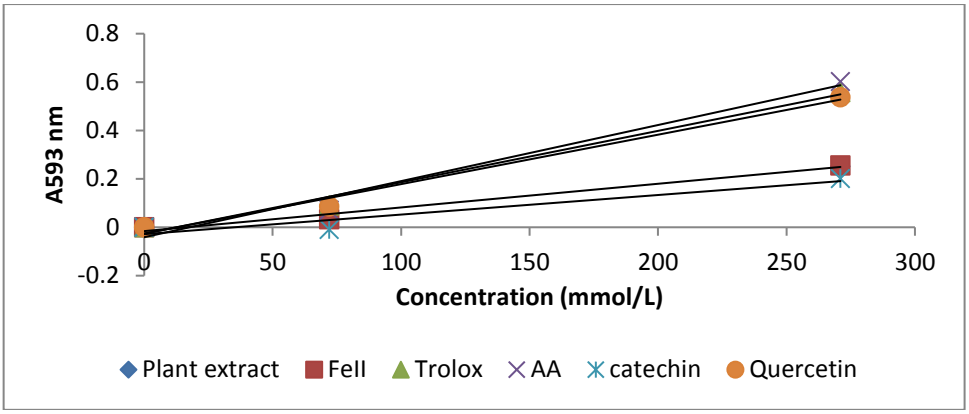
(c)

Figure 4.13 Plot of the dose- response line of the total antioxidant of water extracts of fresh, un-blanched and blanched thyme (4.13 a, b and c respectively), Catechin, Trolox, Ascorbic acid, quercetin and Fe^{II} over the concentration range in the ferric reducing/antioxidant power test (FRAP) assay for reducing (antioxidant) activity. Each point represents the mean of three readings.



(a)

(b)



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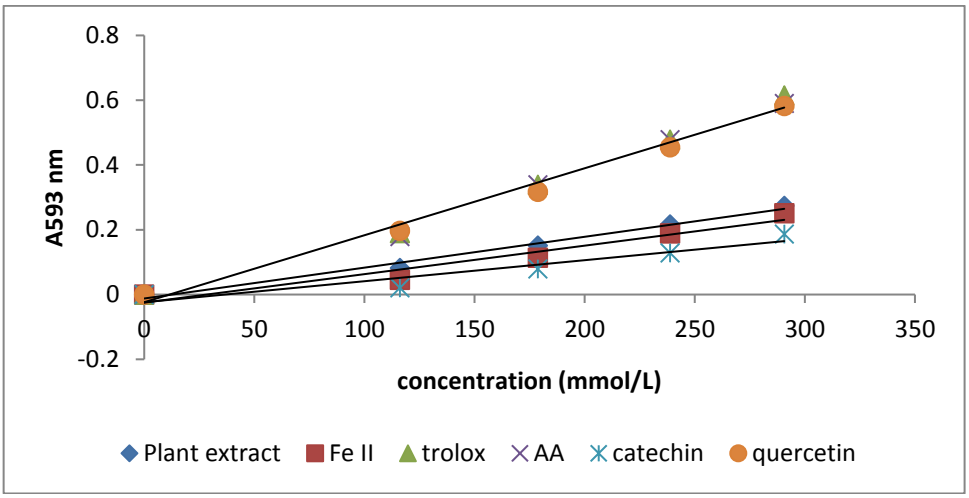


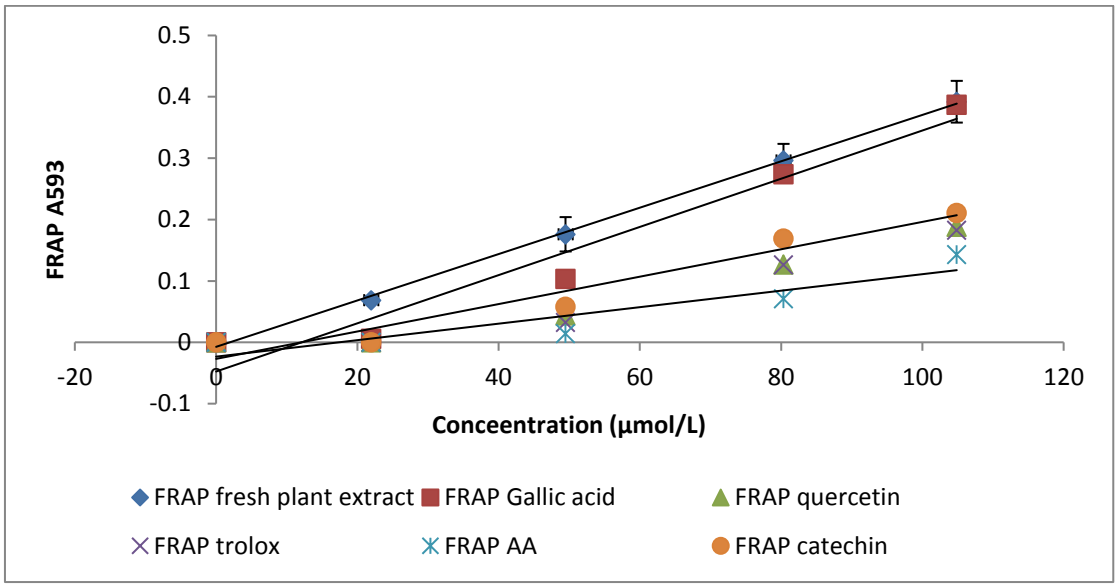
Figure 4.14 Plot of the dose- response line of the total antioxidant of water extracts of fresh, un-blanched and blanched basil (4.14 a, b and c respectively), Catechin, Trolox, Ascorbic acid, quercetin and Fe^{II} over similar concentration range, in the ferric reducing/antioxidant power test (FRAP) assay for reducing (antioxidant) activity. Each point represents the mean of three readings.

4.4.2.1 Comparison between the FRAP of phenolic content of mint extracts and pure phenolic compounds

Figures 4.15 - 4.17 shows the antioxidant efficiency (FRAP) of herb phenolics compared to pure antioxidant compounds. There was no representation of the FRAP of Fe^{II} because Fe^{II} gave very low FRAP signal compared to FRAP of herb extracts and other pure antioxidant compounds within similar concentration range and couldn't be determined. However, the FRAP of herb phenolics consistently showed significantly ($P<0.05$) higher values than FRAP

of Quercetin, trolox and ascorbic acid but remain comparable/similar with gallic acid across all herbs (mint, thyme and basil) and treatments (fresh, un-blanched and blanched frozen).

(a)



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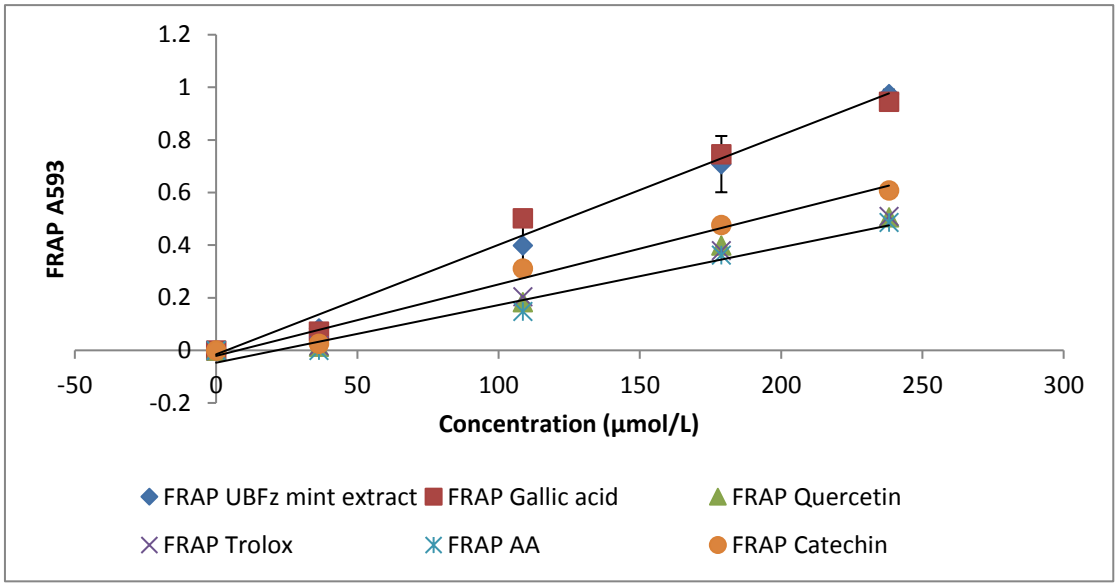
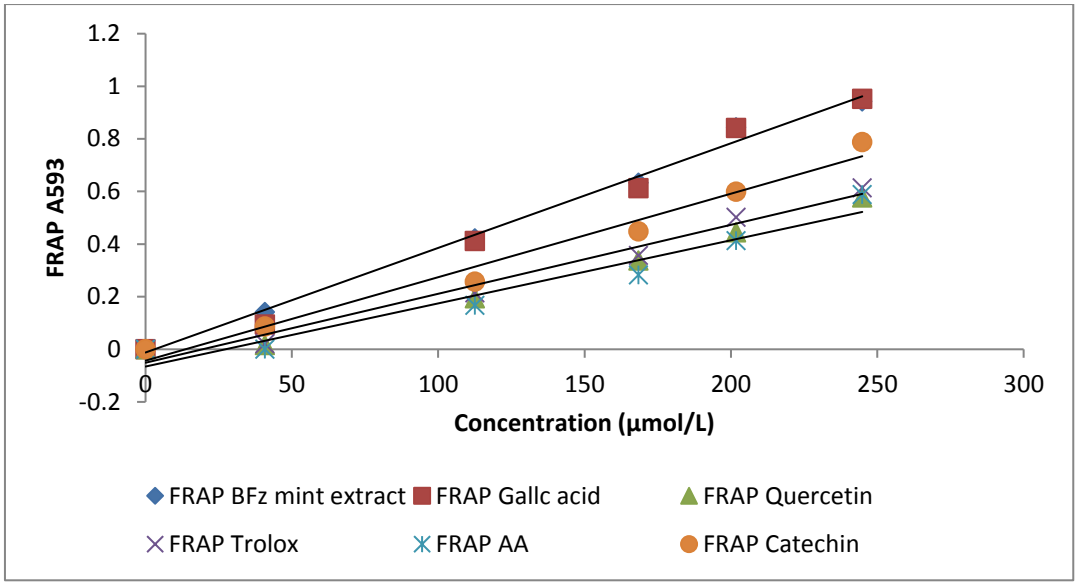
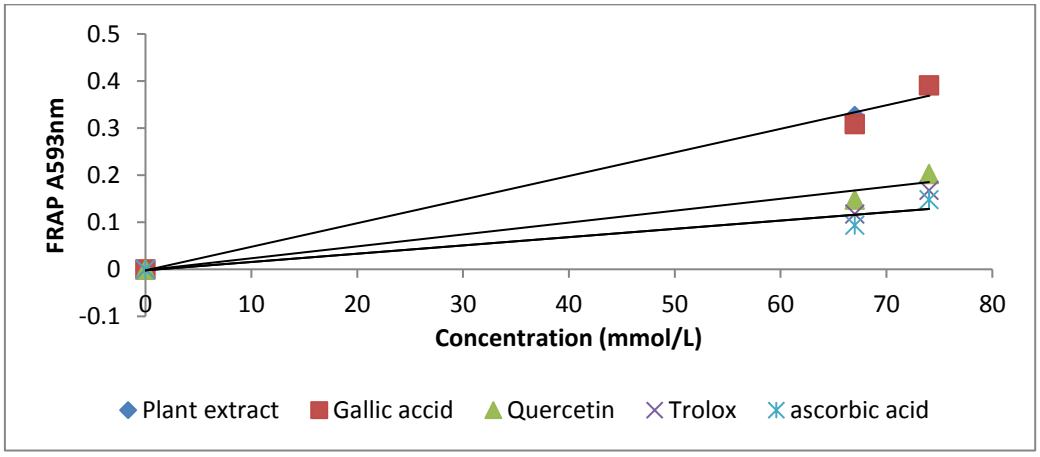


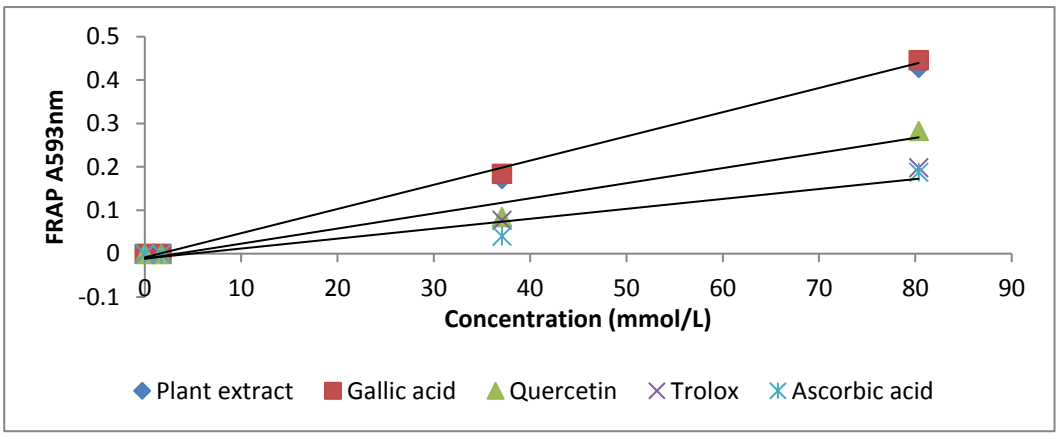


(c)

Figure 4.15 Plot of the dose- response line of the phenolic acid content of water extracts of fresh, un-blanched and blanched mint (4.15 a, b and c respectively), Catechin, Trolox, Ascorbic acid and Quercetin over similar concentration range, in the ferric reducing/antioxidant power test (FRAP) assay for reducing (antioxidant) activity. Each point represents the mean of three readings.



(a)



(b)

(c)

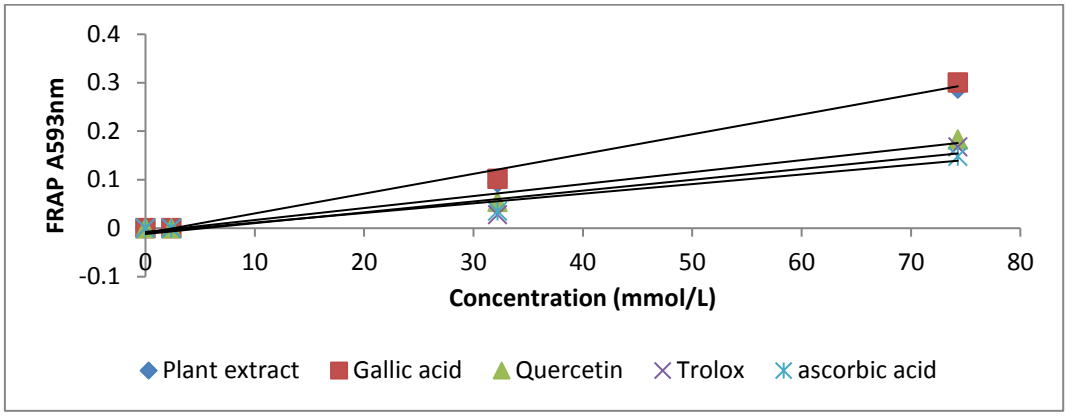
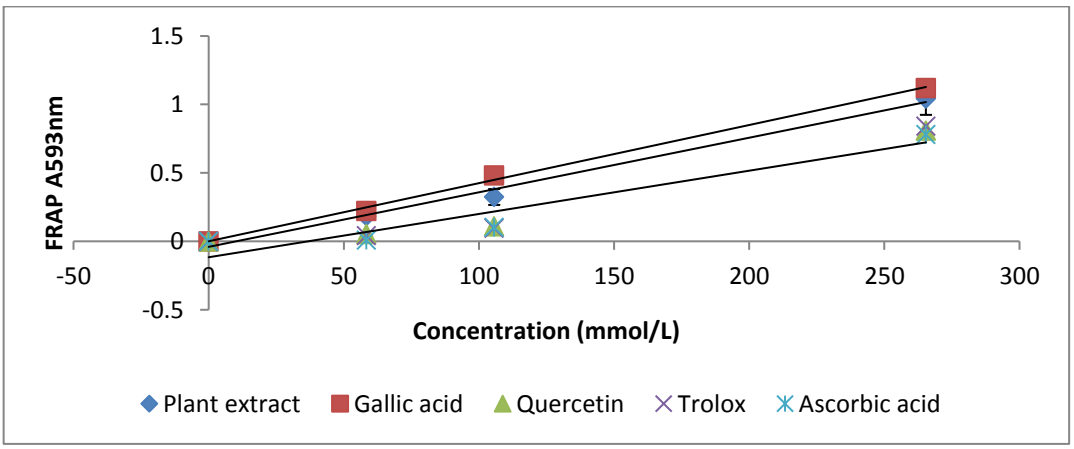


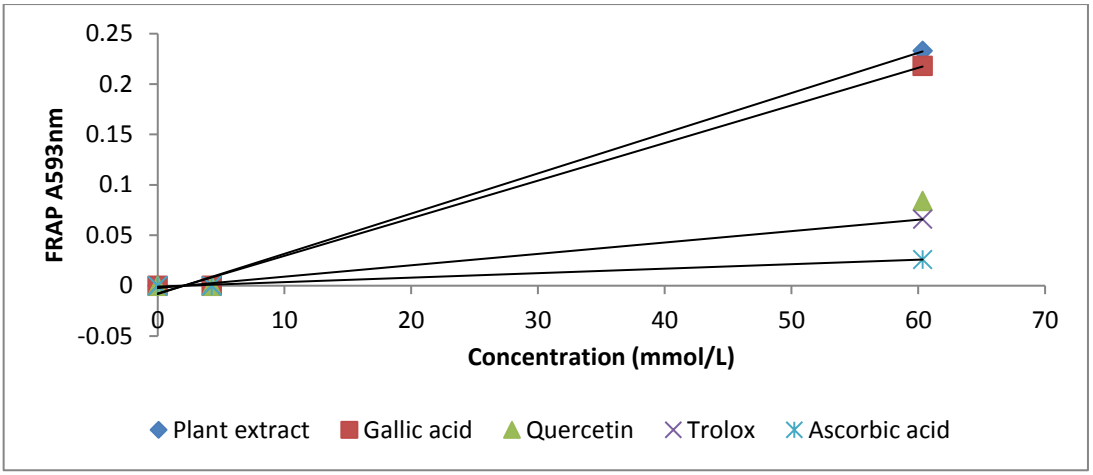
Figure 4.16 Plot of the dose- response line of the phenolic acid content of water extracts of fresh, un-blanched and blanched thyme (4.16 a, b and c respectively), Catechin, Trolox, Ascorbic acid, quercetin and Fe^{II} over similar concentration range, in the ferric reducing/antioxidant power test (FRAP) assay for reducing (antioxidant) activity. Each point represents the mean of three readings

For basil, results of extracts of un-blanched frozen basil (Figure 4.17b) showed better FRAP activity for all assayed synthetic antioxidants compared to extracts of fresh and blanched frozen basil. Furthermore, results of extracts of blanched frozen basil (Figure 4.17c) showed lower FRAP activity than Gallic acid but better than quercetin, trolox and ascorbic acid.

(a)



(b)



(c)

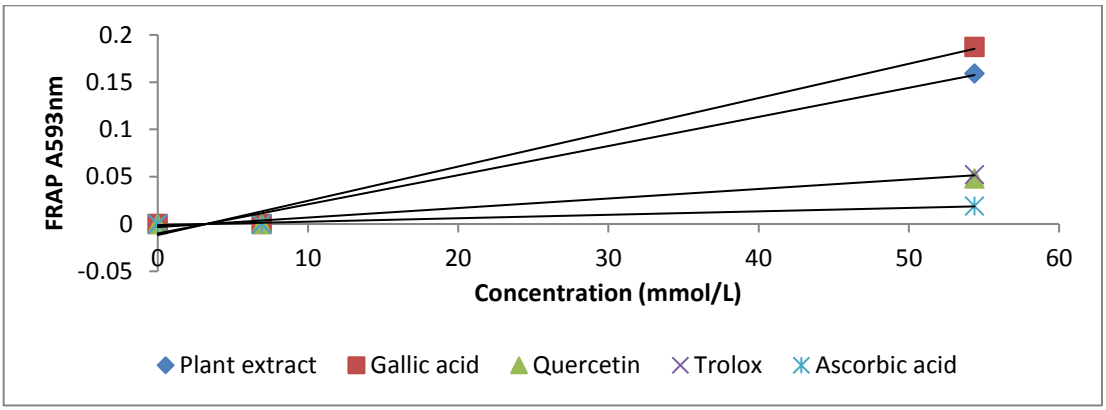


Figure 4.17 Plot of the dose- response line of the phenolic acid ccontent of water extracts of fresh, un-blanched and blanched basil (4.17 a, b and c respectively), Catechin, Trolox, Ascorbic acid, quercetin and Fe^{II} over similar concentration range, in the ferric reducing/antioxidant power test (FRAP) assay for reducing (antioxidant) activity. Each point represents the mean of three readings.

4.5 DISCUSSION

4.5.1 TOTAL ANTIOXIDANT ABILITY OF HERBS MEASURED AS DPPH ACTIVITY.

From the results (Figure 4.1 a, b and c), apart from water extracts of basil (Figure 4.1a), low DPPH inhibition was obtained generally with both blanched and un-blanched herbs, however a significantly (P = 4.25E-05) lower DPPH inhibition was identified in blanched frozen herbs compared to un-branched frozen herbs. The results of DPPH correlate with TPC. Hence it can be deduced that low DPPH results obtained with blanched frozen herbs were due to loss of phenolic compounds via thermal decomposition or leaching of thermal labile and water-soluble

antioxidant. Similarly, low DPPH results in methanol extracts of water blanched white cauliflower has been reported by Ahmed and Ali (2013). However, in contrast Kim, *et al* (2013) reported that there was no significant difference between radical scavenging effect (DPPH) between water extracts of some blanched and fresh medicinal herbs. However, results varied among varieties, species, families, extraction methods and solvents. The low percentage DPPH inhibition obtained with un-blanched frozen herbs may be due to the enzyme activities and the decomposition and subsequent formation of aglycons of polyphenolic and antioxidant compounds with low DPPH reactivity.

Regarding the effects of solvents on the DPPH assay, it has been reported that methanol or ethanol causes no interference with DPPH; hence they work better in DPPH than both water and acetone (Molyneux, 2004). This justifies the high DPPH value obtained with methanolic extracts of herbs.

The results of the CUPRAC values of herbs are not comparable to other studies since there are no previous studies in this research area. However, the loss in CUPRAC values of both blanched and un-blanched frozen herbs suggests the loss of Cu^{2+} reducing antioxidant components of herbs. This may be either through thermal degradation, leaching into blanching water or enzymatic degradation during freezing.

Similarly, methanol extracts of herbs have shown to have higher FRAP value than water extracts. Furthermore, extracts of fresh herbs have shown to be higher than those of blanched and un-blanched frozen herbs. Hence, freezing (blanched and or un-blanched) led to a significant ($P < 0.05$) loss in Fe^{3+} reducing antioxidant components. In contrast, Pujimulyani, *et. al.,* (2012), obtained a higher FRAP value with white saffron, however, samples were prepared differently.

From the results of ORAC, blanching (heat treatment) process had different effects on different herbs, with samples showing either increased or decreased antioxidant activity. This may be linked to their different cell structure/cell compartmentation (Amin and Lee, 2005). Previous studies have also reported varying and inconsistent influences of blanching on antioxidant activities of vegetables such as increased, decreased and stable/insignificant differences (Kim, *et al*, 2013; Wen, *et al*, 2010).

The losses incurred through blanching in hot water may be generally due to huge losses of water soluble (polar) antioxidant/phenolic compounds through leaching and the loss of heat labile nutrients and many bioactive compounds such as members of the flavonoid family (anthocyanins and derivatives of flavan-3-ol). In addition, Shi, *et al* (2003) reported that thermal treatment may lead to the softening of plant tissue hence the weakening of phenol-protein and phenol polysaccharide interactions in plant with resultant loss/transfer of phenols into surrounding solvent (in this case water). Furthermore, heat treatment may lead to the denaturation of cellular membranes resulting to decomposition of phenolic compounds due to hydrolysis, polymerisations and internal redox reactions which may negatively affect quantity, quality and antioxidant activity of bioactive compounds (Huang, *et al*., 2005).

Furthermore, higher antioxidant activities may be attributed to the heat treatment which can lead to the inactivation of oxidative enzymes and other degradative enzymes such as lipoxygenase, polyphenol oxygenase and ascorbic acid oxygenase which leads to the degradation of carotenoids, flavonoids and ascorbic acids respectively (Yamaguchi *et al*, 2003). Consequently, further losses in phenolics and antioxidant compounds are avoided. Additionally, thermal treatment denatures plant cell/vacuoles/matrices leading to easy extractability of cellular bound phytochemicals/phenolics and other antioxidant compounds. Furthermore, thermal treatment can either lead to an increase in antioxidant activities through heat transformation of the glycosides of flavonoids into aglycones, which possess higher antioxidant properties (Turkmen *et al*, 2005).

Increased antioxidant activity obtained with frozen herbs can be explained in a number of ways. In the first place, increased antioxidant activity may be due to the formation of ice crystals which rupture the plant cells hence allowing for improved mass transfer and easy access/penetration of solvent hence enhanced solvent/compound interaction. Furthermore, in the process of attaining freezing temperature (-20˚C), metabolic activity (which continues after plants are harvested) of plants may lead to the accumulation/synthesis of phenolic/antioxidant compounds. This is seen as plants' response to environmental stress such as low temperature, pathogen attack and wounding (Dixon and Paiva, 1995) leading to the synthesis of phenylpropanoid compounds (flavonoids, isoflavonoids, psoralins, coumarins, phenolic acids, lignin and suberin) as a defence mechanism.

The use of the right extraction solvent is one of the most important steps in the study of antioxidants and polyphenolics. Extraction solvents do not only determine the quality and

quantity of polyphenolics and other antioxidants extracted, they also determine the rate of extraction (Xu and Chang 2007). It is not a simple task to select a distinctive solvent for the extraction and analysis of a various group of antioxidants/phenolics in plant material due to the complexity of chemical components with varying chemical structures and properties. However, solvents like methanol, ethanol, acetone, ethyl acetates and their combinations or with different proportions of water are commonly used for the extraction of phenolic compounds and other antioxidant compounds from plant materials due to their distinct specificity in the extraction of polyphenolics. In particular, methanol has been found generally more efficient than other solvents in the extraction of lower molecular weight polyphenols linked to polar fibrous matrices (Chirinos *et al*, 2007; Al Farsi and Lee, 2008) while aqueous acetone is known for the extraction of higher molecular weight flavanols (Prior, *et al*, 2001; Shi, *et al*, 2000) and other polyphenols from protein matrices. In addition to better/higher extractability and recovery of flavanoids such as monomeric flavan-3-ols (catechin and epicatechin) methanol has been reported to be needed to inactivate polyphenol oxidases (Chirinos *et al*, 2007).

For the preparation of food-based extracts, water is deemed the safest, most environmentally friendly and available solvent to use. Due to its polarity, it is expected to aid in the extraction of polar antioxidant compounds and polyphenolic compounds (Turkmen *et al*, 2005).

4.5.2 DOSE RESPONSE LINE OF DPPH OF PHENOLIC ACIDS

Generally, crude water extracts of mint have comparable DPPH scavenging ability with catechin but lower DPPH inhibition than pure gallic acid and quercetin across all treatments. Obviously, quercetin possess high DPPH scavenging inhibition than crude mint extracts. However, crude mint extracts showed higher DPPH scavenging ability than trolox and ascorbic acid. Initial results from this research showed that adequately diluted crude mint extracts of different concentration range calculated as mmol trolox equivalent (mmol TE/L of mint extract) had lower DPPH scavenging ability than all assayed phenolic compounds of similar concentrations but comparable with trolox and ascorbic acid. This result also coincides with report by Katalinic *et al.,* (2004) who reported lower radical (DPPH) scavenging ability of trolox and ascorbic acid. This shows the dominating contribution of phenolic compounds of crude aqueous mint extract in DPPH scavenging ability. Furthermore, the comparable and insignificant difference (R = 0.96) obtained in the antioxidant activity between mint phenolics and (+) catechin in this system coincides with the high catechin content determined by HPLC

(Figure 4.5). However, this was not affected by the loss in (+) catechin upon freezing (blanched and un-blanched). Furthermore, this can also be explained with the insignificant difference ($p > 0.05$) between the total phenolic content (TPC) of fresh and un-blanched frozen aqueous extracts and un-blanched and blanched frozen herbs. Freezing may have led to formation of aglycons or esters of (+) catechin which have scavenging ability. On the other hand, scavenging effect has been reported as to depend on the number of free hydroxyl groups in the molecule, which are said to be strengthened by steric hindrance (Dziedzic and Hudson, 1983). Hence, hydroxycinnamic acids such as ferulic and caffeic acids were found to be more effective than their hydroxybenzoic acid (gallic acid) counterparts, possibly due to the aryloxy-radical stabilizing effect of the –CH=CH–COOH linked to the phenyl ring by resonance (Rice-Evans *et al*., 1996).

Furthermore, the scavenging ability of un-blanched and blanched frozen mint extracts may have been due to hydroxycinnamic acid such as chlorogenic and p-coumeric acids which were detected only in frozen (un-blanched and blanched) (Figure 4.5). These hydroxycinnamic acids are said to be covalently bound to plant cells structures such as cellulose, lignin, pectin and structural proteins (Wong, 2006). However, they can be liberated by food processes such as freezing, thermal treatment, fermentation and alkaline hydrolysis. Furthermore, when liberated these phenolic acids have been reported to possess greater antioxidant activities measured as DPPH and ABTS, than other phenolic compounds (Bhanja *et al*., 2009). Hence high DPPH inhibition ability of extracts from frozen mint may be due to liberation of cellular bound chlorogenic and p-coumeric acids.

4.5.3 THE DOSE-RESPONSE OF FRAP OF HERB EXTRACT AND INDIVIDUAL PHENOLIC ACID

From the results, FRAP of herb phenolics consistently showed significantly ($P = 3.62E-04$) higher values than FRAP of Quercetin, trolox and ascorbic acid but remain comparable/similar with gallic acid across all treatments (fresh, un-blanched and blanched frozen). However, comparability differed slightly with freezing (un-blanched and blanched). That is to say that, on freezing (un-blanched and blanched), FRAP values of phenolic compounds of mint extracts became less comparable with gallic acid (Figures 4.15b and c respectively). This coincides with results of individual phenolic compounds (Figure 4.5) with the decrease in

hydroxybenzoic acid on freezing (blanched and un-blanched) of mint. Furthermore, just like DPPH scavenging, FRAP values also depends on the structure of phenolic and antioxidant compounds. Khoker and Apentent (2003) proposed that optimum metal-binding and antioxidant activity is associated with structures which contain hydroxyl-keto group (a 3-OH or 5-OH and a 4-C=O) as well as large number of gallol and catechol group. The loss in comparability of frozen (un-blanched and blanched) crude extracts with gallic acid and near comparability with (+) catechin may be due to the liberation of bound phenolic compounds which are comparable to catechin.

Generally, the FRAP values of quercetin, catechin, trolox and ascorbic acid remained comparable. Results from Benzi *et al*., (1996) and Katalinic *et al*, (2004) reported comparable FRAP activity between ascorbic acid and trolox. However, Katalinic *et al.* (2004) reported that quercetin had higher FRAP value than catechin trolox and ascorbic acid. The difference may be due to difference in methods and parameters such as sample incubation temperature and time. Furthermore, apart from difference in FRAP values of quercetin and catechin, the FRAP of Gallic or any other hydroxybenzoic acid in comparison with other phenolic and antioxidant compounds has never been reported.

4.6 CONCLUSION

Generally, it can be concluded that selected *lamiceae* herbs are good sources of antioxidant displaying good oxygen radical absorption capacity (ORAC) and their ability to scavenge radicals (DPPH). Furthermore, the herbs showed good ability to reduce reactive metal ions Fe^{3+} and Cu^{2+} to non-reactive metal ions Fe^{2+} and Cu^{+} in the FRAP and CUPRAC assay, respectively.

Furthermore, both CUPRAC and FRAP assays showed significant reduction in antioxidant activity of methanol extracts of herbs when frozen (blanched and un-blanched). Hence, there is loss or degradation of Cu^{2+} and Fe^{3+} reducing antioxidant components during blanching and or freezing of herbs. Therefore, for better CUPRAC and FRAP of herbs for domestic use and herbal treatments, herbs are better used fresh.

In some cases, for DPPH and ORAC assays, there was either significant increase or decrease in the total antioxidant capacity of herbs in different solvents (water and methanol) and different treatments (fresh, blanched and un-blanched frozen). This therefore gives hints as to

the necessary solvent or treatments necessary to obtain maximum and or better antioxidant yield or value for domestic use or herbal treatments.

CHAPTER 5

USE OF AQUEOUS EXTRACTS OF HERBS IN THE CONTROL OF POSTPRANDIAL BLOOD GLUCOSE AND HYPERTENSION - EFFECTS OF DOMESTIC FREEZING (-20˚C) TEMPERATURE AND BLANCHING PRIOR TO FREEZING ON THE ENZYME INHIBITION FUNCTIONALITIES OF LAMIACEAE HERBS

5.1 INTRODUCTION

The origin of type II insulin-independent diabetes mellitus and correlating morbidities such as cardiovascular diseases, hypertension obesity and hyperlipidemia has been linked to hyperglycemia a condition characterized by an abnormal postprandial increase of blood sugar (Haffner, 1998; Dicarli, *et al*., 2003; Sowers, *et al*. 2001).

Alpha-amylase is an endo- acting enzyme found around the digestive organs which specifically catalyses the hydrolysis the 1- 4-α-D glucosidic linkages of starch, amylos, amylopectine, glycogen and several maltodextrines to maltose and finally to glucose which is the only useable sugar in the body absorbed in the small intestine (Kotowaro, *et al*, 2006).

Due to the role of α-amylase in the breakdown of carbohydrates, absorption of glucose with subsequent increase in postprandial blood glucose leading to type II diabetes, the inhibition of α-amylase has been deemed necessary. The inhibition of α-amylase has been made possible using substances referred to as α-amylase inhibitors.

Alpha glucosidase is an enzyme that catalyses the hydrolysis of carbohydrates to glucose which is easily absorbed into the body. In disease conditions like non-insulin dependent diabetes or type II diabetes, excess increase in postprandial blood glucose can lead to further health problems. Hence the control or inhibition of α-glucosidase will help to delay the absorption of glucose after a meal.

Just like α-amylase, several researchers have reported the α-glucosidase inhibition ability by several plants photochemical (Jaiswal *et al*., 2012; Kumar *et al*., 2011; Kwon *et al*., 2006; Prinya Wongsa *et al*., 2012), however all reported works were on dried herbs. No work has been carried out comparing the effects of freezing on the enzyme inhibition nor has there been any report on fresh herbs.

One major complication of diabetes is high blood pressure or hypertension. Angiotensin converting enzyme (ACE) is an important enzyme involved in the maintenance of vascular tension. ACE activates agiotensin I (a dipeptide of histidyl-leucine) into a potent angiotensin II which is a vasoconstrictor. Angiotensin II is also known to stimulate the synthesis and release of aldosterone which promotes sodium retention in the distal tubules hence increasing blood pressure (Sowers *et al*, 2001). Hence the inhibition of angiotension I-converting enzyme is seen as a very useful tool for therapeutic treatment of high blood pressure in both diabetic and non-diabetic patients.

Rosemary and lemon balm (herbs of *Lamiceae* family) have been tested and found to inhibit angiotensin I-converting enzyme (Kwon *et al*, 2006). However, no work has been done on angiotensin I-converting enzyme inhibition using assayed herbs and the treatments in this thesis.

Analysed individual phenolic acids were chosen based on previous reports on the phenolic acid content of *Lamaiceae* herbs and their enzyme inhibition effectiveness (Cheplick, *et al.*, 2010; Kwon, *et al.*, 2006; Wongsa, *et al.*, 2012). Kwon *et al* (2006) reported that the enzyme inhibitory effects were attributed to plant phenolic compounds such as catechin (99.1%), caffeic acid (91.3%), rosmarinic acid (85.1%), resveratrol (71.1%), catechol (64.4%), protocatecheuic acid (55.7%) and quercetin (36.9%).

The main aim of this research is to screen three well known and commonly used culinary herbs of the *lamiceae* family (Mint, Thyme and Basil) for the control of postprandial blood glucose and hypertension, to test their ability to inhibit two carbohydrate hydrolysing enzymes (α-amylase and α-glucosidase) and angiotensin I-converting enzyme.

Furthermore, effects of freezing and blanching treatments of the herbs on α-amylase, α-glucosidase and ACE inhibition activity was tested and compared using spectrophotometry and HPLC.

5.2 RESULTS OF ENZYME INHIBITION

The enzyme inhibition ability of herbs was carried out as described in Chapter 2 (section 2.2.9).

The HPLC detection of un-hydrolysed hippuryl-histidyl-Leucine (HHL) and absence of hippuric acid (HA) and histidine leucine indicated that assayed herbs did not show any inhibitory action towards angiotensin converting enzyme across all treatments. The HPLC chromatograms of the assayed herbs were compared to that of a control (Captopril) using hippuric acid and histidine leucine as standards.

The Summary of the α-amylase and α-glucosidase inhibition assay is shown in Figures 5.1a and b. Tested herbs showed different levels of enzyme inhibition across all treatments.

Generally, there was a low α-amylase inhibition activity across all herbs and treatments, however, extracts of fresh herbs showed to have the highest inhibition compared to extracts of un-blanched and blanched frozen samples. Furthermore, results also showed that fresh thyme extracts possess a significantly (P = 2.05E-06) lowest α-amylase inhibition (5.16%) compared to mint (12.79%) and basil (11.53%). It was also observed that blanching prior to freezing reduced the α-amylase inhibition of extracts of all herbs.

Results of α-amylase inhibition activity of mint extracts showed that the highest inhibition is observed with fresh mint while extracts of blanched frozen samples showed the least inhibition effects. An ANOVA of result data showed a significant difference (P = 4.92E-09) between the inhibition effects of all extracts. A further post hoc t-test showed a significant difference between inhibition effects of fresh and un-blanched frozen (P = 1.77E-04), and blanched (P = 3.11E-08) frozen samples.

For the α-amylase inhibition activity of extracts of thyme, extracts of fresh samples showed the highest inhibition compared to un-blanched and blanched frozen extracts while blanched frozen samples showed the least inhibition effect. An ANOVA of result data showed a significant difference (P = 1.59E-06) between inhibition effects of extracts of all samples, however, there is no significant difference (P = 0.44) between inhibition effects of fresh and un-blanched frozen samples. Furthermore, there were significant differences between the inhibition effects of extracts of fresh and blanched frozen (P = 2.05E-04), and that of un-blanched and blanched frozen (P = 1.99E-05) samples.

For the α-amylase inhibition activity of exracts of basil, extracts of fresh sample showed the highest α-amylase inhibition effects compared to extracts of frozen samples while extracts of blanched frozen samples shows the least. The ANOVA of result data showed a significant difference (P = 6.21E-08) between α-amylase inhibition of extracts of all samples. A further post hoc t-test showed a significant difference between inhibition effects of extracts of fresh and those of un-blanched frozen (P = 1.99E-06), and blanched frozen (P = 7.12E-06) samples. Furthermore, there was also a significant difference (P = 6.03E-04) between the inhibition effects of extracts of un-blanched and blanched frozen samples.

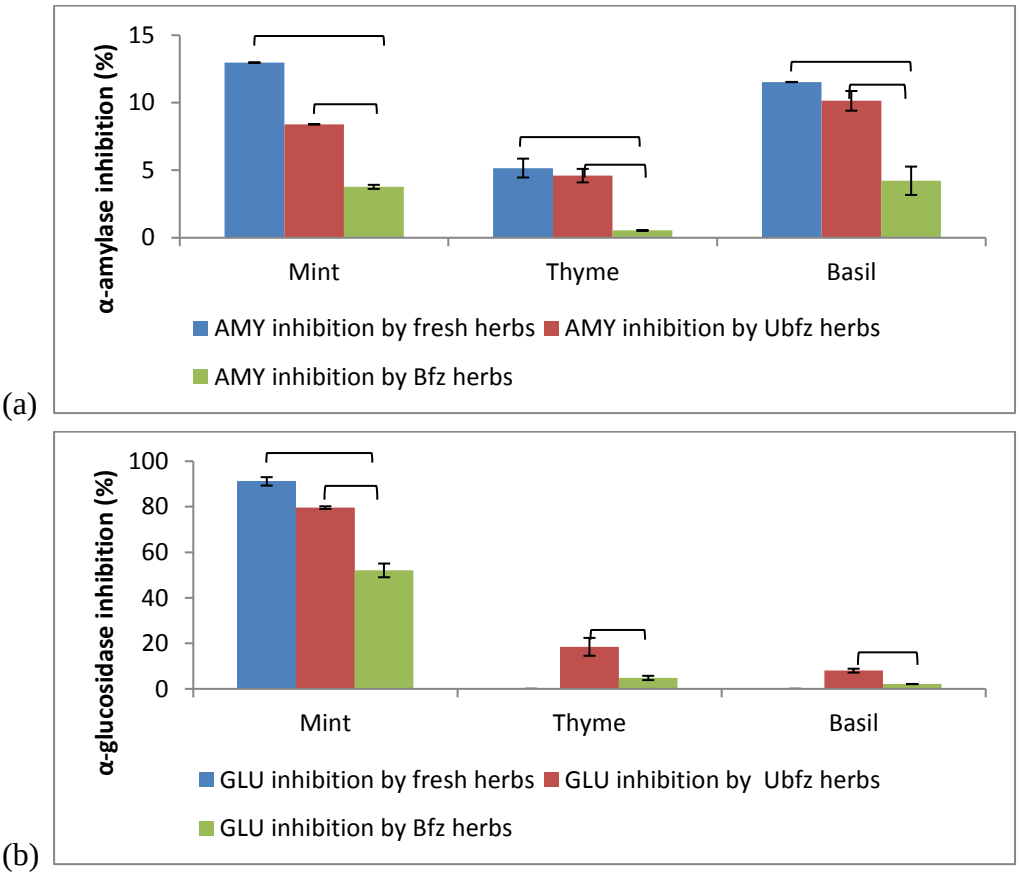


Figure 5.1 Plot of the representation of the α-amylase and α-glucosidase inhibition of fresh, un-blanched frozen (Ubfz) and blanched frozen (Bfz) mint, basil, thyme. Results are means of three readings. Values linked with ⊓ are significantly different (P<0.05)

α-glucosidase activity was assessed by the release of p-nitrophenol (pnP) by PnPG *in vitro.* The result of the α-glucosidase inhibition (Figure 5.1b) shows that mint generally possesses the highest α-glucosidase inhibition than other herb extracts. Furthermore, among tested

extracts of all fresh herbs, thyme and basil showed no α-glucosidase inhibition while mint showed 8.85% release of pnP hence α-glucosidase inhibition of 91.15 %. In contrast, the α-glucosidase inhibitory effect of thyme and basil were only evident in un-blanched and blanched frozen.

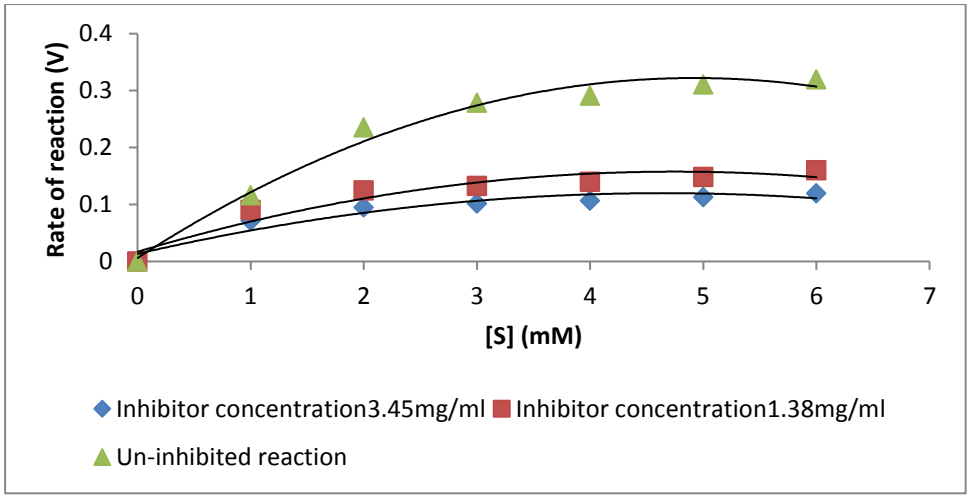
Results of the α-glucosidase inhibition activity of mint, extract of fresh samples showed the highest inhibitory effect while the extracts of blanched frozen sample showed the least effect. AN ANOVA of result data showed a significant difference (P = 3.18E-08) between inhibitory effects of extracts of all samples. A further post hoc t-test confirmed a significant difference between inhibitory effects of extracts of fresh samples and those of un-blanched frozen (P = 1.25E-03), and that of blanched frozen (P = 1.09E-06) samples.

As earlier stated, for thyme and basil, the α-glucosidase inhibitory effects were only observed with extracts of un-blanched and blanched frozen samples, with extracts of un-blanched frozen samples having a significantly higher inhibitory effects than those of blanched frozen sample (P = 8.01E-09 for thyme, and P = 1.11E-06 for basil).

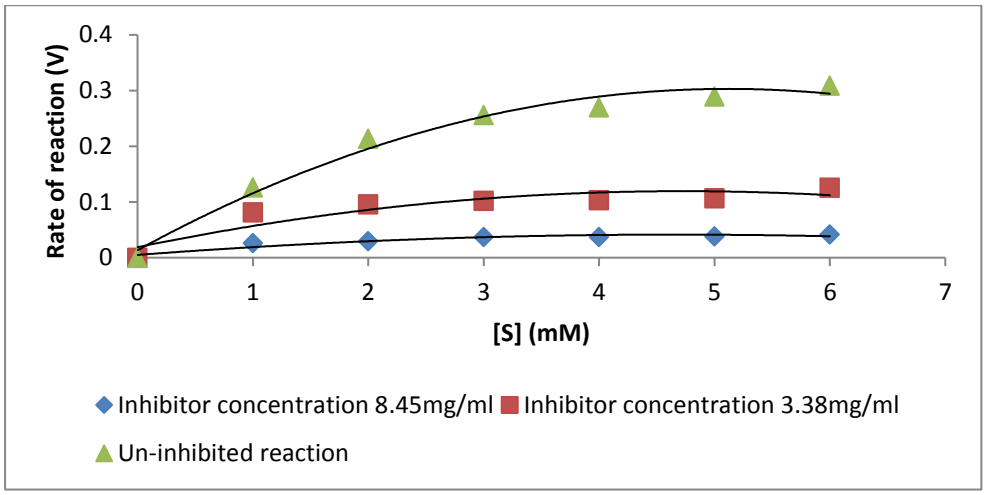
5.3.1 ENZYME INHIBITION KINETICS

Further investigations were carried out on mint extracts due to its enzyme inhibition activity. Figure 5.2 shows the plot of the rate of reaction of enzyme inhibition of fresh (Figure 5.2a), un-blanched frozen (Figure 5.2b) and blanched frozen (Figure 5.2c) mint extracts in the presence of different concentrations of p-nitrophenyl-α-D-glucopyranoside (1, 2, 3, 4, 5 and 6 mM) as substrate. The figure clearly shows that rate of inhibition decreases with substrate concentration across all treatments. However, at different sample concentrations, fresh and un-blanched samples showed better inhibition (Figures 5.2a and 5.2b) compared to blanched frozen samples (Figure 5.2c).

(a)



(b)



(c)

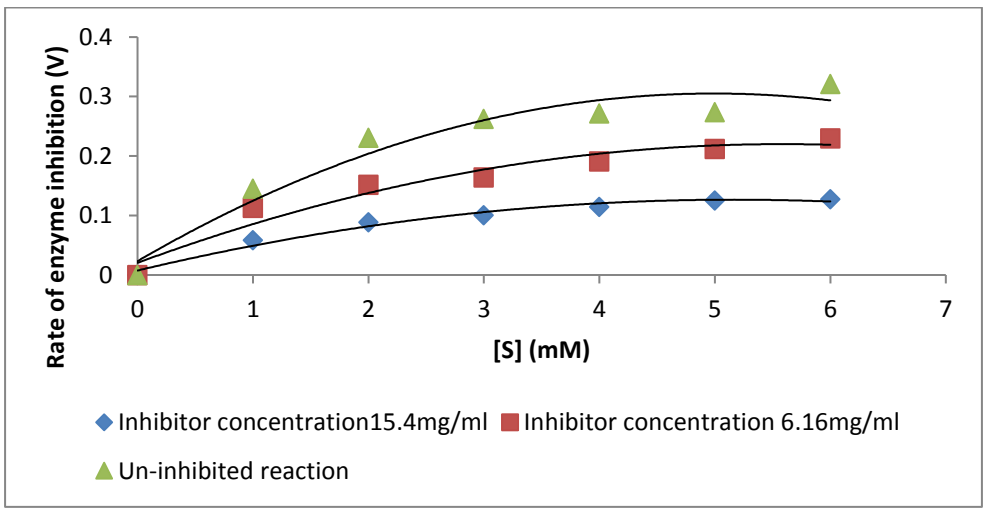


Figure 5.2 Plot of the rate of reaction of S. Cerevisiae α-glucosidase inhibition in PnPG with fresh (a), un-blanched frozen (b) and blanched frozen (c) mint extracts in the presence of different concentrations of pNPG.

5.3.2 Lineweaver-Burke plot of S. cerevisiae α-glucosidase inhibition by mint extracts in the presence of PnPG as subtrate

The results of enzyme inhibition kinetics of mint extracts demonstrated by the Lineweaver-Burk plot of the inverse of reaction rate (1/v) versus inverse of different pNPG substrate concentrations (1/[s]). The Lineweaver-Burke plot of fresh mint extracts exhibits a typical un-competitive inhibitory activity. This is shown as sets of parallel lines of inhibited reactions and un-inhibited reactions with different intercept at the 1/V (y) and 1/[s] (x) axis (Figure 5.3).

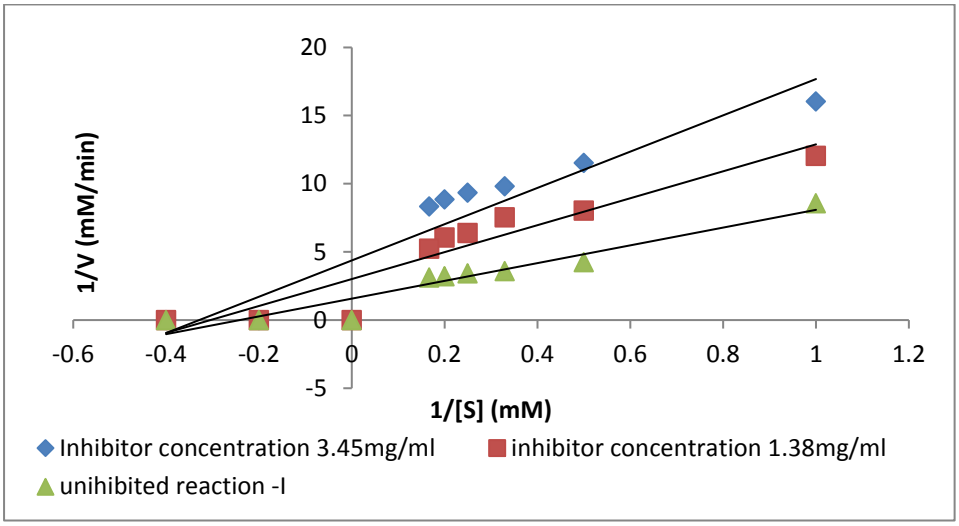


Figure 5.3 Plot of the Lineweaver-Burk enzyme kinetic of the inhibition of *s.cerevisiae* α-glucosidase in PnPG by fresh mint extract.

Extract concentration (mg/ml)	V_{max} (mM/min)	K_m (mM)	IC_{50} (mg/ml)
0	0.64^{a}	4.17^{b}	
1.38	0.33^{a*}	3.28^{b*}	0.77 ± 0.02
3.45	0.23^{a*}	3.03^{b*}	

Table 5. 1 Kinetic analysis of α-glucosidase inhibition by fresh mint extracts.

***In the presence of inhibitor (fresh plant extracts) V_{max} and K_m become $V_{max\ apparent}$ and $K_{m\ apparent}$ respectively; Values with similar superscript alphabets are significantly different (P < 0.05).**

The kinetics of α-glucosidase inhibition by fresh mint extract is presented in Table 5.1. From the table, in the presence of inhibitor (fresh plant extract), there was a decrease in the maximum velocity (V_{max}) of reaction and the enzyme affinity constant (K_m) of the inhibitor. Furthermore, an increase in the extract concentration of fresh mint extract (1.38mg/ml to 3.45mg/ml) brought about a further decrease of 0.10mM/min in V_{max} with subsequent significant ($P < 0.05$) decrease in K_m (3.20 to 3.08 mg/ml). Furthermore, the IC_{50} of fresh mint extracts were determined to be 0.77mg/ml.

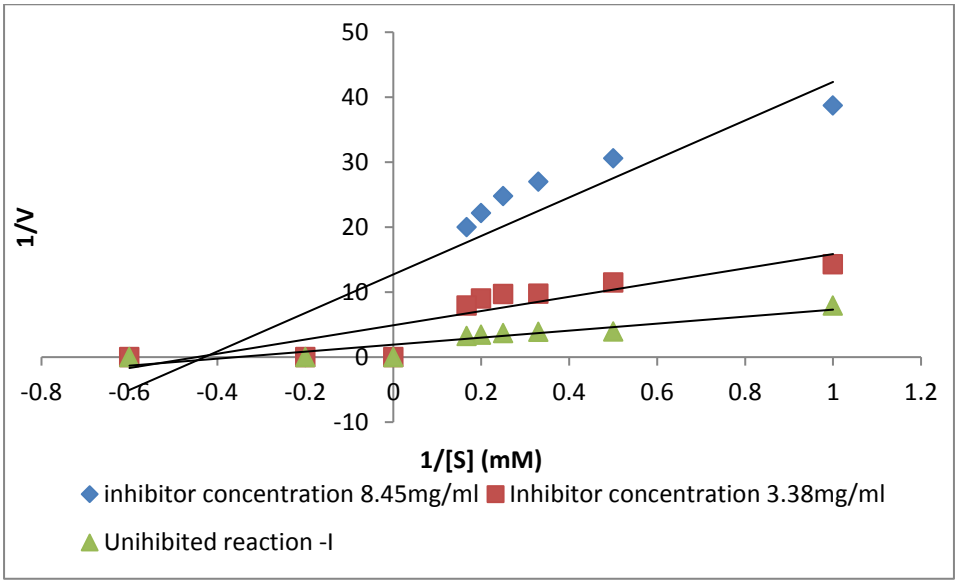


Figure 5.4 Microsoft Excel plot of the Lineweaver-Burk enzyme kinetic of the inhibition of *s.cerevisiae* α-glucosidase in PnPG by un-blanched frozen mint extract.

Extract concentration (mg/ml)	V_{max} (mM/min)	K_m (mM)	IC_{50} (mg/ml)
0	0.52	2.76 [b]	
3.38	0.20[a]*	2.22*	0.65 ± 0.03
8.45	0.08 [a]*	2.33 [b]*	

Table 5.2Kinetic analysis of α-glucosidase inhibition by un-blanched frozen mint extracts.

***In the presence of inhibitor (un-blanched frozen plant extracts) V_{max} and K_m become $V_{max\ apparent}$ and $K_{m\ apparent}$ respectively; Values with similar superscript alphabets are significantly different**

Similarly, un-blanched frozen herbs showed un-competitive inhibition to α-glucosidase (Figure 5.4) with an IC_{50} of 0.65mg/ml. From the enzyme inhibition kinetics represented in Table 5.2, the presence of extracts of un-blanched frozen mint (3.38mg/ml) led to a significant (P = 1.51E-04) reduction in V_{max} and $K_{m\ apparent}$ (0.52mM/min to 0.20 mM/min and 2.76 mg to 2.22 mg respectively). Furthermore, an increase in extract concentration (3.38 to 8.45 mg/ml) brought about a further decrease in $V_{max\ apparent}$ (0.20 mM/min to 0.08mM/min) with a very slight insignificant (P = 0.08) increase in $K_{m\ apparent}$ (2.22mg to 2.33mg). Hence an increase in concentration of un-blanched frozen does not significantly change $K_{m\ apparent}$.

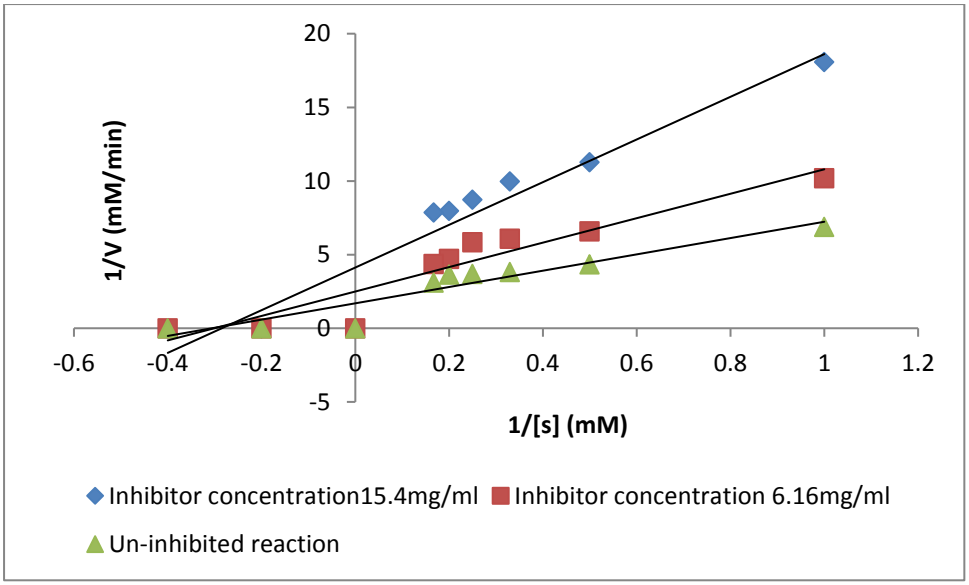


Figure 5.5 Microsoft Excel plot of the Lineweaver-Burk enzyme kinetic of the inhibition of *s.cerevisiae* α-glucosidase in PnPG by blanched frozen mint extract.

Extract concentration (mg/ml)	V_{max} (mM/min)	K_m (mM)	IC_{50} (mg/ml)
0	0.59 [a]	3.23 [b]	
6.16	0.40[a]*	3.33 [b]*	0.89 ± 0.07
15.40	0.24 [a]*	3.51 [b]*	

Table 5. 3 Kinetic analysis of α-glucosidase inhibition by blanched frozen mint extracts.

***In the presence of inhibitor (blanched frozen plant extracts) V_{max} and K_m become $V_{max\ apparent}$ and $K_{m\ apparent}$ respectively; Values with similar superscript alphabet are significantly different (P < 0.05)**

The IC_{50} of blanched frozen mint extracts was determined to be 0.89mg/ml. The Lineweaver-Burke plot of enzyme inhibition by blanched frozen mint extract (Figure 5.5) shows a typical mixed inhibition activity. The result (Table 5.3) shows a decrease in V_{max} and an increase in K_m in the presence of inhibitor source (blanched frozen mint extract). On further increase in inhibitor concentration (6.16mg/ml to 15.40mg/ml), there was a decrease in $V_{max\ apparent}$ (0.40mM/min to 0.24 mM/min) with an increase in $K_{m\ apparent}$ (0.33mg to 3.51mg) respectively. This conforms to a typical mixed inhibitor mode of reaction which has been reported to bind

to free and to substrate bound enzyme and interfere with binding and catalysis of substrate, increasing the enzyme affinity constant ($K_{m\ apparent}$) and decreasing reaction rate $V_{max\ apparent}$ (Cornish-Bowden, 2013). The influence of the inhibitor over binding of substrate to enzyme is caused either by the nearness of the binding sites of inhibitors and substrates to each other or the conformational changes in enzymes caused by the inhibitor which in turn affects the binding of the substrate.

5.4 RESULTS OF HPLC PHENOLIC ACID (PA) PROFILE OF EXTRACTS

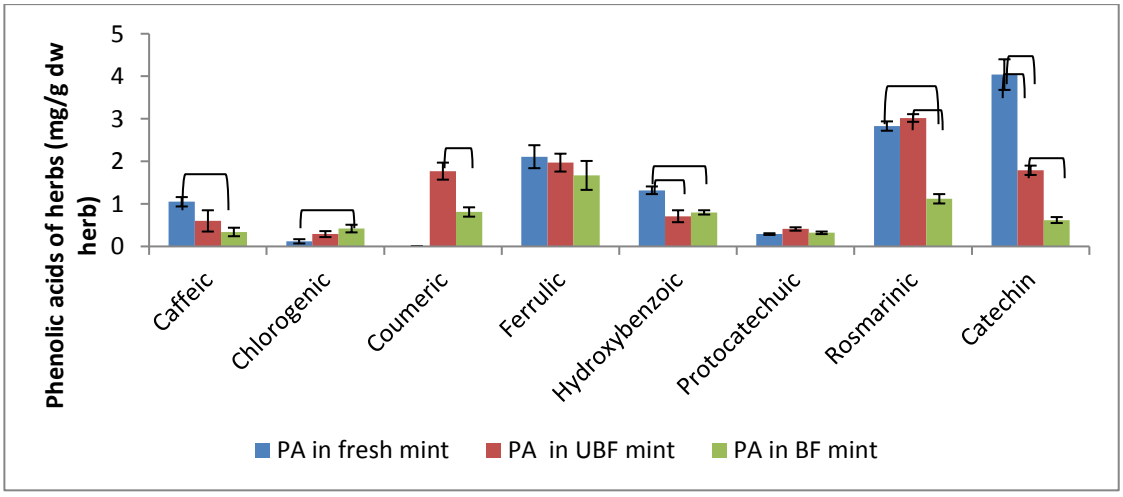
Results of the HPLC profile of phenolic compounds extracts used for enzyme inhibition are presented in Figures 5.6 a (mint), b (Thyme) and c (Basil). Results varied across herbs and treatments.

Mint contained significantly high to trace amount of all analysed PA apart from coumeric acid, which was totally absent in fresh mint. Fresh mint seemed to contain significantly highest amount of catechin compared to other PA across all treatments.

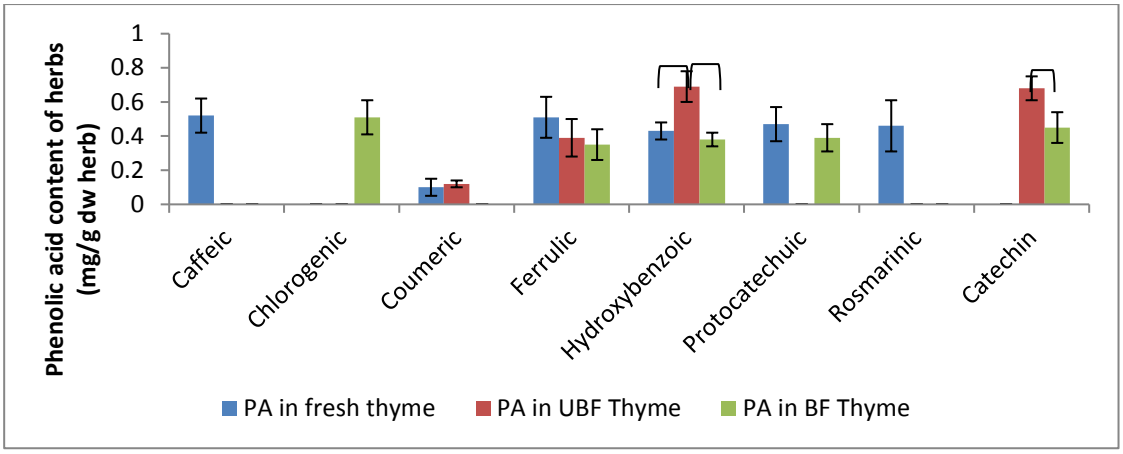
Analysed thyme showed varying phenolic acid content. Apart from ferrulic acid and hydroxybenzoic acids, which were identified in all treatments, other phenolic acids were either present or absent in different treatments.

Results from basil showed that apart from rosmarinic and catechin, which were both absent on blanched frozen samples, and coumeric acid (only present in un-blanched frozen sample), all other PA were significantly present in either high or trace quantity across all treatments.

(a)



(b)



(c)

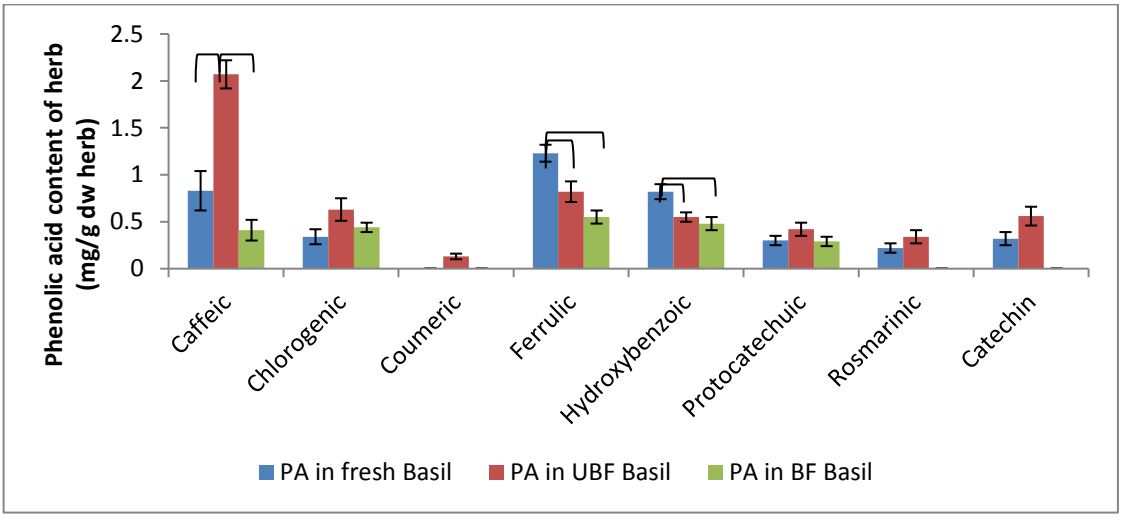


Figure 5.6 Plot of the representation of the HPLC determination of individual phenolic compounds of fresh, un-blanched frozen (UBF) and blanched frozen (Bf) (a) mint, (b) thyme and (c) basil extracts. Results are means of three readings of three different experiments. Values linked with ⌈⌉ are significantly different (P<0.05).

From the results in Figure 5.6, mint possesses the highest phenolic acid content which corresponds to its high enzyme inhibition effectiveness. The phenolic acid content of blanched frozen extracts of mint and basil were lower than those of fresh and un-blanched frozen herb extracts (4.54 and 0.97 mg total analysed phenolic acids/g dw herbs respectively) which corresponds to their enzyme inhibition effectiveness (52.04 and 2.11 % respectively). In contrast, with thyme, in as much as the enzyme effectiveness of blanched thyme extracts are very low (18.46%), there is no significant difference between its phenolic acid content (1.12mg of total analysed phenolic compounds/g of herb) and those of fresh (1.96mg of total analysed phenolic compounds/g of herb) and un-blanched frozen extracts (1.11mg of total analysed phenolic compound/g of herb).

5.5 Correlation between determined individual phenolic compounds and the results of enzyme inhibition assay.

The relationship between individual phenolic acid content of herbs and the enzyme inhibition effective is represented by Pearson's correlation matrix (Tables 5.4 – 5.6). For the fresh herb extracts (Table 5.4), there is a strong positive correlation between α- amylase and α-glucosidase inhibition and caffeic acid (R = 0.94 and R = 0.87 respectively), ferrulic acid (R = 0.89 and 0.92 respectively) and p-hydroxybenzoic acid (R = 0.85 and 0.95 respectively). Consequently, caffeic acid, ferrulic acid and p-hydroxybenzoic acids may be taken as strong contributors to both α-amylase and α-glucosidase inhibition of fresh herb extracts. Wongsa *et al* (2012) also reported a strong positive correlation between caffeic acid and α –amylase inhibition effect (R = 0.68) and a low correlation with α-glucosidase inhibition effect (R =0.28). Furthermore, there was a high/weak positive correlation between α-amylase and rosmarinic acid (R = 0.57) and catechin (R = 0.66). In contrast very strong positive correlation exists between α-glucosidase and rosmarinic acid and catechin (R = 1.00 each). Hence rosmarinic acid and catechin may be seen as weak and strong contributors to the α-amylase and α -glucosidase inhibition of fresh herb extracts respectively. This is concurring with a report by Kwon *et al* (2006) who reported high α–glucosidase inhibition of rosmarinic acid and catechin (85.1% and 99.1% respectively). In contrast, there was low to negative correlation between chlorogenic, coumeric and protocatechuic acids and both α-amylase and α-glucosidase inhibition effectiveness, which can be said to be non-contributors to enzyme inhibition effectiveness of fresh herb extracts. This coincides with the report of Wongsa *et al* (2012) who reported correlation (R = 0.33) of α-glucosidase with p-coumeric acid. However, there was no previous report for chlorogenic, ferrulic and p-hydroxybenzoic acid.

For the un-blanched frozen herbs (Table 5.5), caffeic acid (R = 0.92), chlorogenic acid (R = 0.98) and protocatechuic acid (R = 0.98) a high positive correlation with α-amylase inhibition effectiveness but a low to negative correlation with α-glucosidase inhibition (R = -0.31,0.27 and 0.29 for caffeic, chlorogenic and protocatechuic acids respectively). On the other hand, low to negative correlations were obtained between coumeric acid (R = 0.19), catechin (R = 0.36), Ferrulic acid (R = 0.21), rosmarinic acid (R = 0.28), hydroxybenzoic acid (R = -0.27) and α-amylase while high positive correlation was obtained with α-glucosidase (R = 0.99, 0.96, 0.99, 0.94 and 0.98 for coumeric, catechin, ferrulic, rosemarinic and p-hydroxybenzoic acids respectively.

	Caff	*Chl*	*Cou*	*Fer*	*Hydbz*	*Protc*	*Ros*	*Catc*	*α-AMY*	*α-GLU*
Caff	1									
Chl	0.13	1								
Cou	-0.87	-0.61	1							
Fer	0.99	-0.02	-0.79	1						
Hydbz	0.98	-0.06	-0.75	1.00	1					
Protc	-0.44	-0.95	0.83	-0.32	-0.26	1				
Ros	0.82	-0.46	-0.42	0.89	0.92	0.15	1			
Catc	0.88	-0.36	-0.52	0.93	0.95	0.04	0.99	1		
α-AMY	0.94	0.46	-0.98	0.89	0.85	-0.72	0.57	0.66	1	
α-GLU	0.87	-0.38	-0.50	0.92	0.95	0.06	1.00	1.00	0.64	1

Table 5. 4 Pearson's correlation coefficients of phenolic compounds. Remarks: Caffeic acid-Caff, chlorogenic acid-Chl, coumeric acid- Cou, ferrulic acid- Fer, p-hydroxybenzoic acid –Hybdz, protocatechic acid- Protc, rosmarinic acid- Ros, catechin-Catc) and enzyme inhibition effectiveness (α- amylase- α-AMY and α-glucosidase-α-GLU) of fresh herb extracts

	Caff	*Chl*	*Cou*	*Fer*	*Hydbz*	*Protc*	*Ros*	*Catc*	*α-AMY*	*α-GLU*
Caff	1									
Chl	0.83	1								
Cou	-0.21	0.38	1							
Fer	-0.18	0.40	1.00	1						
Hydbz	-0.62	-0.08	0.89	0.88	1					
Protc	0.82	1.00	0.39	0.42	-0.06	1				
Ros	-0.11	0.47	0.99	1.00	0.84	0.48	1			
Catc	-0.02	0.54	0.98	0.99	0.80	0.56	1.00	1		
α-AMY	0.92	0.98	0.19	0.21	-0.27	0.98	0.28	0.36	1	
α-GLU	-0.31	0.27	0.99	0.99	0.94	0.29	0.98	0.96	0.06	1

Table 5. 5 Pearson's correlation coefficients of phenolic compounds. Remarks: Caffeic acid-Caff, chlorogenic acid-Chl, coumeric acid- Cou, ferrulic acid- Fer, hydroxybenzoic acid –Hybdz, protocatechic acid- Protc, rosmarinic acid- Ros, catechin-Catc) and enzyme inhibition effectiveness (α- amylase- α-AMY and α-glucosidase-α-GLU) of un-blanched frozen herb extracts

	Caff	*Chl*	*Cou*	*Fer*	*Hydbz*	*Protc*	*Ros*	*Catc*	*α-AMY*	*α-GLU*
Caff	1									
Chl	-0.24	1								
Cou	-0.22	0.89	1							
Fer	0.31	0.91	1.00	1						
Hydbz	-0.67	0.85	1.00	0.99	1					
Protc	0.22	0.88	0.58	0.61	0.50	1				
Ros	-0.34	0.90	1.00	1.00	1.00	0.51	1			
Catc	0.99	0.99	0.84	0.86	0.79	0.93	0.84	1		
α-AMY	0.98	-0.04	0.40	0.37	0.49	-0.51	0.40	-0.16	1	
α-GLU	0.17	0.92	1.00	1.00	0.99	0.62	1.00	0.87	0.36	1

Table 5. 6 Pearson's correlation coefficients of phenolic compounds. Remarks: Caffeic acid-Caff, chlorogenic acid-Chl, coumeric acid- Cou, ferrulic acid- Fer, hydroxybenzoic acid –Hybdz, protocatechic acid- Protc, rosmarinic acid- Ros, catechin-Catc) and enzyme inhibition effectiveness (α- amylase- α-AMY and α-glucosidase-α-GLU) of blanched frozen herb extracts

Table 5.6 shows the Pearson's correlation matrix for phenolic acids individual phenolic acids and enzyme inhibition effectiveness of blanched frozen herbs. The table shows positive high correlation between α-amylase and caffeic, coumeric, hydroxybenzoic, rosmarinic acids (R = 0.98, 0.40, 0.49, 0.40 respectively) and a low to negative correlation with chlorogenic (R = 0.04), ferrulic (R = 0.37), protocatechuic acid (R = -0.51) and catechen (R = -0.16). Meanwhile, apart from caffeic acid which showed low positive correlation with α-glucosidase inhibition (R = 0.17) the rest of the phenolic acids showed very high positive correlation ranging from 0.62 to1.00.

5.3.3 DISCUSSION

This present study focused on investigating the potential effects of *Lamiaceae* herbs (mint, thyme and basil) to inhibit key carbohydrate hydrolysing enzymes such as pancreatic α-amylase, *S.cereviseae* α-glucosidase and ACE.

As previously stated, assayed herbs and treatments do not possess ACE inhibition ability. Kwon, et al (2006), have reported the inhibition of ACE by some clonal herbs of the *Lamiceae* family such as lemon balm and rosemary. There has been no report on the ACE inhibition properties of basil and thyme, neither has there been any report comparing the effects of blanching and freezing on the ACE inhibition of selected herbs.

The enzymes α-amylase and α-glucosidase which are key carbohydrate hydrolysing enzymes are responsible for the production of glucose after a carbohydrate meal by breaking α,1-4 bonds in disaccharides and polysaccharides (Rhabasa-Lhorete and Chiasson, 2004). The release of glucose after a carbohydrate rich meal contributes to hyperglycaemia, the main trait of type II diabetes mellitus (DM). The ability of plant extracts to modulate glucose liberation from starch and its subsequent absorption has proved to be an attractive therapeutic means of managing type 2 DM.

From the investigation, all analysed *Lamiaceae* herbs showed considerable alpha amylase inhibition with the highest inhibition obtained with fresh mint extract (12.79%) followed by fresh and un-blanched frozen basil extracts (11.53% and 10.14% respectively. Blanched frozen thyme extracts had the least inhibition activity. However, it can be observed that blanching significantly reduced the α-amylase inhibitory activity of all herbs (3.77%, 4.22% and 0.54% for mint, basil and thyme respectively) as against fresh (12.79%, 11.53% and 5.16% for mint, basil and thyme respectively) and un-blanched frozen herbs (8.4%, 10.14% and 4.6% for mint, basil and thyme respectively). From these results, blanching prior to freezing significantly ($P < 0.05$) affects the α-amylase inhibition activity of mint, basil and thyme while freezing without blanching does not significantly affect the α-amylase inhibition activity of investigated *Lamiaceae* herbs. This may be due to either the loss of water soluble or thermal liable porcine pancreatic α-amylase (PPA) inhibiting compounds of herbs during blanching. Furthermore, freezing without blanching does not have significant effect on the concentration of PPA inhibiting compounds. Several researches have reported on the low to no PPA inhibiting activity of several common herbal plants (Kwon, et al, 2006; Nikavar, *et al*, 2008; Wongsa, *et al*, 2012; Sudha, *et al*, 2011), however there has been no report on the effects of home freezing (-20˚C) and blanching prior to home freezing (-20˚C) on the PPA inhibiting activity.

The results of the α-glucosidase inhibition clearly show that the investigated *Lamiaceae* herbs are effective species for the management of post-prandial hyperglycemia. However, mint is significantly ($P = 4.27E-05$) the most effective amongst all analysed herbs across all treatments (fresh, un-blanched and blanched frozen herbs). Further analysis with mint showed that fresh mint possesses the highest α-glucosidase inhibition activity followed by un-blanched frozen then the blanched frozen herbs. However, the enzyme inhibition kinetics showed that un-blanched frozen mint extracts possess the most effective inhibitors with a low IC_{50} of 0.65 mg/ml followed by inhibitors of fresh extracts (IC_{50} 0.77mg/ml) with blanched having the least

effective inhibitors (IC_{50} 0.89mg/ml). The mode of inhibition of extracts show uncompetitive inhibition for both fresh and un-blanched frozen herbs while blanched frozen extracts showed mixed inhibition.

The un-competitive inhibition of α-glucosidase by components of fresh and unblanched frozen mint extracts implies that inhibition components of these mint extracts bind exclusively to the enzyme-substrate (E-S) complex yielding an inactive enzyme-substrate-inhibitor complex (Bacchawat *et al*., 2011). This complex is reported to reduce affinity for the enzyme active site for the substrate and hence decreases the affinity and delays rate of reaction (Cornish-Bowden, 2013). Furthermore, an uncompetitive inhibitor can't interact with enzyme alone but with the E-S complex in a reversible reaction. Hence with uncompetitive inhibition, the lowest inhibition is found with the lowest substrate concentration and the highest inhibition is found with the highest substrate concentration. Hence, we can say that the more the substrate concentration the more the inhibition.

The mixed inhibition mode of blanched frozen extracts means that the inhibitors present may bind to the enzyme whether or not the enzyme has already bound to the substrate but has a greater affinity for one state or the other. This may be due to the conceptual mixture or presence and synergistic action of both competitive and un-competitive inhibiting constituents.

Since correlation coefficients of variables show the relationship between different variables, from the results, it can be deduced that for different treatments (fresh, un-blanched frozen and blanched frozen herbs) the phenolic acids with low to negative correlation with different enzyme inhibition may not be responsible for inhibition effectiveness of that enzyme. On the other hand, phenolic acids with high positive correlation with different enzyme inhibitions indicate that those phenolic acids participated strongly in the inhibition effectiveness of that particular enzyme inhibition.

In addition, it must be stated that the varying and inconsistent results obtained between the individual phenolic acid content and the enzyme inhibition assays goes to show that the enzyme inhibition effectiveness of individual phenolic acid may not only depend on the relative concentrations of phenolic acids but also on the phenolic acid profile (quality).

5.4 CONCLUSION

Generally, it can be concluded that all investigated herbs irrespective of treatment contain inhibitors which inhibit PPA to a safe level such that may avoid some stomach disturbances which is common with acarbose such as abdominal gaseous distention, flatulence and in some cases, diarrhoea. However, since fresh herbs showed the highest but moderate inhibition against PPA and blanching significantly reduced PPA inhibition ability, it is worth considering using fresh herbs for herbal treatment/control of postprandial blood glucose in the case of alpha amylase inhibition.

Furthermore, to obtain the highest α-glucosidase inhibition, fresh mint shows a better choice than other assayed herbs and treatment. However, with a significantly low IC_{50} of 0.65 mg/ml, un-blanched frozen herbs contain the most effective inhibitor, and consequently should be considered over fresh and blanched. Furthermore, the treatment choice given to herb prior to use for herbal control of postprandial blood glucose should depend on the mode of inhibition (competitive, un-competitve or mixed inhibition) required.

Generally, for mint, blanching seemed to give the poorest/lowest results, hence for the purpose of control of postprandial blood glucose through α-glucosidase and α-amylase inhibition, blanching should be avoided. In contrast to mint, freezing and blanching may be the only option to obtain a good result for the control of postprandial blood glucose through α-glucosidase and α-amylase inhibition for both thyme and basil.

CHAPTER 6

EFFECTS OF DOMESTIC FREEZING (-20˚C) TEMPERATURE AND BLANCHING PRIOR TO FREEZING ON THE PREBIOTIC FUNCTIONALITIES OF LAMIACEAE HERBS.

6.1 INTRODUCTION

Probiotics can be either non-pathogenic normal gastrointestinal bacterium found in the microflora of healthy individuals or bacterial preparations that have been clinically proven to impact beneficial clinical effects on the health of hosts when already present or consumed. *Lactobacillus* and *Bifidobacteria* cultures are the main strains used as probiotics in pharmaceuticals and in foods.

The strain *Lactobacillus rhamnosus* is an aerobic homofermentative lactic acid producing bacteria and is generally regarded as "safe" (Berry *et al*., 1997). Strains of *L. rhamnosus* have been linked to several health promoting effects, one of which is a long-term protective effects against eczema in infants of 2 years (Wicken *et al*., 2013). On the other hand, *Bifidobacteria bifidum* a non-motile, non-spore forming bacteria is anaerobic and is known to produce acetic acid, ethanol, formic acid and succinic acid. Many health benefits have been equally attributed to *B.bifidum*. Generally all strains are known to have anti-inflammatory properties (Presing *et al*., 2010), some have been reported to have shown the ability to inhibit the binding of pathogenic *E.coli* (Fujiwara *et al*., 1997) and alleviate global irritable bowel syndrome (Guglielmetti *et al*., 2011).

Reports have also stated that although probiotic bacteria can survive transit in the gastrointestinal tract, they do not colonize and grow during shorter or after prolonged feeding periods (Klaenhammer, 2000; Tannock *et al*., 2000). Consequently, there is a need for complementary means to promote/maintain growth in the colon.

Prebiotics are dietary factors specifically intended to promote/stimulate the growth of probiotic bacteria (Grizard and Barthomeuf, 1999). The most common prebiotic compound used and reported by researchers which is known to promote the growth of probiotic bacteria is the non-digestible oligosaccharide *inulin* (Kaplan and Hutkins, 2000; Rao, 1999). However, it has been reported that the disadvantage of the use of carbohydrate type prebiotic is that they also promote the growth of non-probiotic bacteria. Bello *et al*. (2001) have demonstrated that the use of fructo-oligosaccharides (FOS) resulted in enhanced growth of *Eubacterium biform* and

Clostridum perinfringes. Hence there is search for a non-carbohydrate source of prebiotic compounds.

Consequently, some studies have studied and reported the prebiotic functions of products with high phenolic compound content. For instance, in a study, Goto *et al.* (1998) found that the administration of 300 mg of catechin per day to 15 subjects over a 3-week period significantly increased faecal levels of lactobacilli and bifidobacteria with a significant decrease in levels of *Enterobacteriaceae*, *Bacteroidaceae* and *Eubacteria*. Furthermore, Ishihara *et al*. (2001) evaluated and reported the inhibition of pathogenic bacteria by green tea while encouraging and maintaining intestinal microflora balance in calves by high faecal counts of Bifidobacterium species and Lactobacillus species. Some researchers have also worked and reported on the prebiotic effects of selenium and selenium containing green tea (Molan *et al*., 2008). Hence from these past reports, catechin which is the main phenolic component of tea and selenium has been implicated as alternative prebiotic compounds. However, no work has been carried out on the prebiotic function of herb extracts in relation to their selenium and different phenolic content.

Hence, the aim of this study was to: -

- Evaluate the antioxidant activity measured as DPPH inhibition ability and phenolic compound content of aqueous extracts.
- Evaluate the effects of aqueous extracts of selected common culinary herbs of *Lamiaceae* family to act as prebiotic to enhance the viability and growth of beneficial lactic acid bacteria using pure cultures of *L. rhamnosus* and *B.bifidum*.
- Find the correlation between these prebiotic effects to selenium and different phenolic compound contents of extracts.

6.2 RESULTS

6.2.1 TOTAL PHENOLIC ACID CONTENT AND ANTIOXIDANT ACTIVITY OF HERBS

The results of the total phenolic content and total antioxidant activity (DPPH scavenging ability) of herb extracts are presented in Figures 6.1 and 6.2 respectively.

Just like in Chapters 3 and 4 (total phenolic content and total antioxidant assays), herb extracts showed considerable total phenolic content and total antioxidant activity (DPPH scavenging ability). However, results varied from herbs (mint, thyme and basil) and treatments (fresh, un-blanched and blanched frozen). Generally mint extracts possess the highest total phenolic content (TPC) compared to thyme and basil. However, freezing without blanching (un-blanched frozen) led to 28% and 48% more TPC compared to fresh and blanched frozen samples respectively while blanching prior to freezing led to 33% less TPC compared to fresh samples. Similarly, extracts of thyme also showed that un-blanched frozen samples possess the highest TPC, more than fresh and blanched frozen samples (8% and 46% respectively) while blanched prior to freezing showed 41% less TPC compared to fresh samples. In contrast, extracts of basil showed that un-blanched frozen samples possessed 74% and 30% more TPC than both fresh and blanched frozen samples respectively, while blanched frozen showed 63% more TPC than fresh samples.

Furthermore, extracts of mint showed the highest total antioxidant activity (DPPH) compared to thyme and basil. However, just like TPC aqueous extracts of un-blanched frozen mint showed 23% and 28% higher DPPH scavenging ability than extracts of fresh and blanched frozen mint respectively while extracts of blanched frozen samples showed 12% lower DPPH scavenging ability than extracts of fresh samples. Although there is difference in scavenging ability with extracts of different herb treatments there was no significant difference ($P = 0.48$) between extracts of fresh and un-blanched frozen mint. This shows that extracts of un-blanched frozen mint possess similar antioxidant activity (% scavenging effect) compared with fresh mint. Hence, after bulk buying of mint herbs, home freezing storage (-20˚C) of 1 week will make no significant difference in its antioxidant activity functions. In contrast, blanching prior to freezing leads to a significantly ($P = 2.77E-03$) lower antioxidant capacity (DPPH scavenging) compared to un-blanched samples.

Aqueous extracts of fresh thyme showed the highest DPPH scavenging ability compared to extracts of both un-blanched and blanched samples; 32% and 47% higher respectively. While

extracts of blanched frozen samples showed a significantly lower DPPH scavenging ability compared to fresh samples.

Aqueous extracts of blanched frozen basil samples on the other hand showed the highest DPPH scavenging ability compared to extracts of fresh and un-blanched frozen samples basil. The result shows a significant (P = 1.13E-05) 72% and non-significant (P = 0.07) 14% higher scavenging ability compared to fresh and un-blanched frozen samples respectively.

Generally, similar effects of blanching and freezing were obtained in results of the TPC and total antioxidant activity (DPPH scavenging ability) of aqueous extracts of herbs from the assays in Chapters 3 and 4.

There has not been any report on the effects of freezing and blanching on the TPC and total antioxidant activity measured as DPPH scavenging ability of the investigated herbs (mint, thyme and basil). However, researchers such as Kmiecik *et al*., (2007) investigated and reported that blanching at 95-98˚C during a period of 3-5mins and immersion cooking significantly led to significant decrease in some minerals such as potassium, phosphorous, sodium and magnesium in broccoli. Gliszczynska *et al*. (2006) also reported that thermal treatment of broccoli increased the total amount of some bioactive compounds such as glucosinolates. However, the increase was attributed to extractability rather than an increase, hence the bioavailability of these compounds. Furthermore, Gebczynski and Lisiewska (2006) investigated and reported that blanching prior to freezing freezing negatively affected the polyphenol content and antioxidant activity of broccoli compared to un-blanched frozen vegetables. They further reported a steady decrease in the properties along storage period of 12months at -20˚C to -30˚C. However, freezing has been reported to encourage extractability of some cellular bound compounds. This is made possible by the formation of ice crystals and the rupturing of plant cells leading to easy solvent penetration.

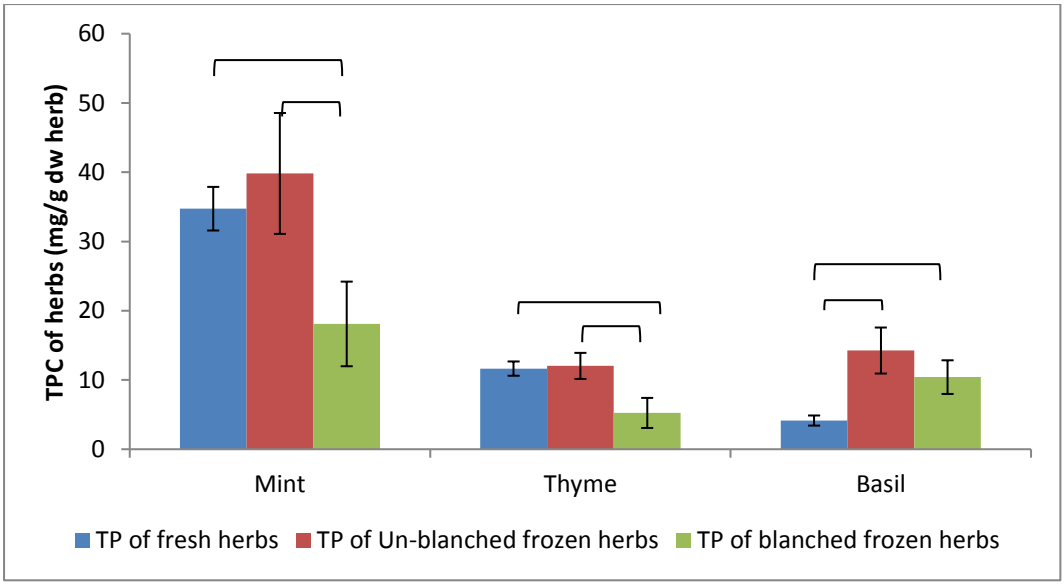


Figure 6.1 Plot of the representation showing the effects of blanching and freezing on the total phenolic content of extracts from fresh, un-blanched and blanched frozen herbs. Values linked with a ∩ are significantly different (P < 0.05)

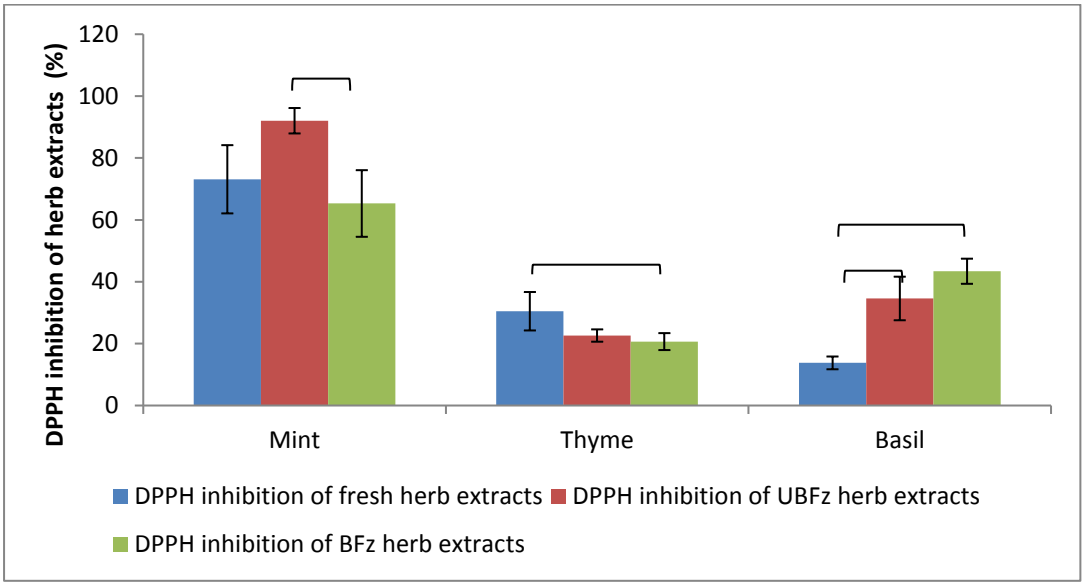


Figure 6.2 Plot of the representation showing the effects of blanching and freezing on the DPPH inhibition (%) of extracts from fresh, un-blanched and blanched frozen herbs. Mint; Thyme and Basil used for prebiotic assay. Values linked with a ∩ are significantly different (P < 0.05)

6.2.2 PHENOLIC COMPOUNDS OF HERB EXTRACTS

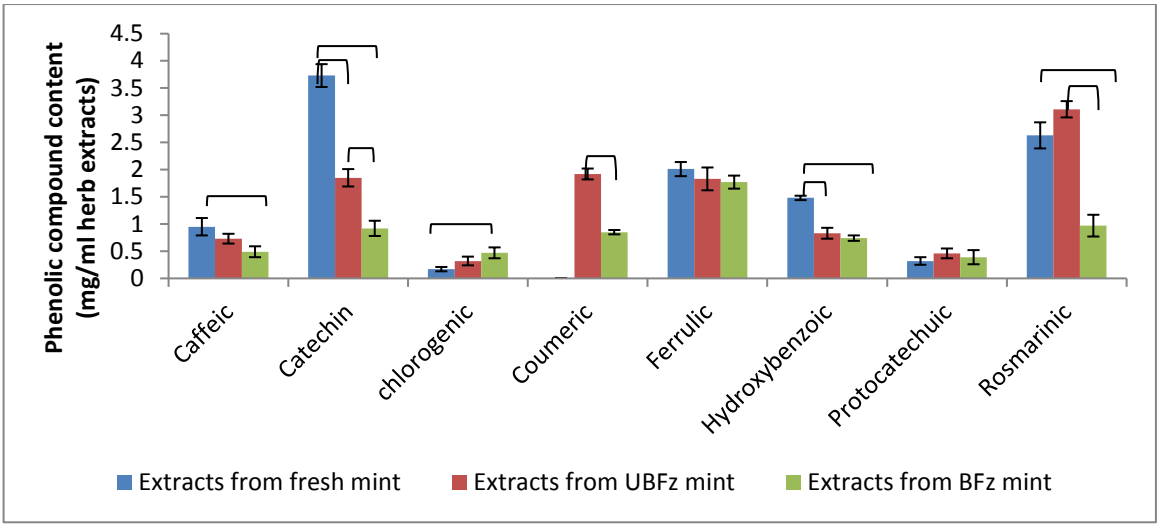
Results of HPLC assays of individual phenolic compound contents of herb extracts are presented in Figures 6.3 a, b and c. Generally, phenolic compounds varied with herbs and treatments.

All mint extracts (fresh and frozen) were shown to possess all assayed phenolic compounds apart from coumeric acid which was only detected in extracts from frozen mint. Catechin was generally the highest phenolic compound detected in all extracts of mint, however, reduced on freezing (un-blanched and blanched). Generally, apart from ferrulic and protocatechuic acids which showed no significant difference ($P = 0.38$) between treatments, concentration of all other assayed phenolic compounds either reduced or increased on freezing. On freezing without blanching (un-blanched frozen) this showed lower caffeic acid (23%), catechin (50%), ferrulic (9%) and hydroxybenzoic acids (44%) compared to fresh samples. On the other hand, significantly higher yield was obtained with coumeric acid (100%) chlorogenic acid (46%), protocatechuic acid (40%) and an insignificant 18% increase in rosmarinic acid. Furthermore, apart from a higher chlorogenic acid content (32%), blanching prior to freezing yielded lower caffeic acid (32%), catechin (50%), coumeric acid (29%), ferrulic acid (3%), Hydroxybenzoic acid (10%), protocatechuic acid (15%) and rosmarinic acid (68%) compared to un-blanched frozen samples.

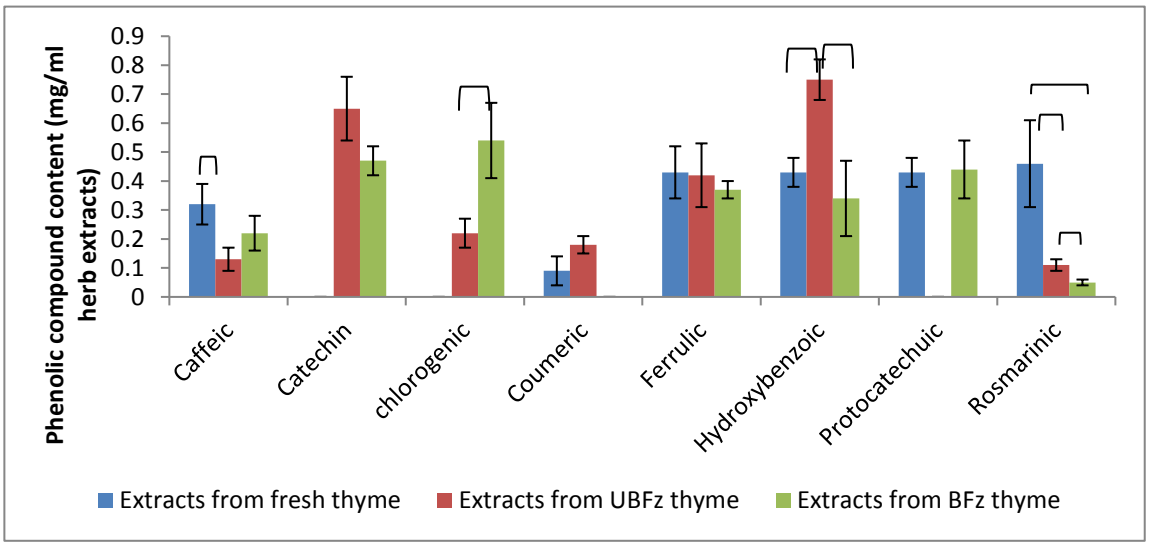
A similar assay was carried out on aqueous extracts for enzyme inhibition (Chapter 5). However different results were obtained for different phenolic compounds and different herb treatments.

From these results, phenolic compound content and extractability from different herbs depends on the structure and the nature of the herbs. Furthermore, different phenolic compounds behave differently under different storage conditions. For instance, Chlorogenic and p-coumeric acids are hydroxycinnamic acids which occur in bound forms in plant cells such as lignin, cellulose, hemicelluloses, pectin and rod-shaped structural proteins (Wong, 2006). Hence freezing/blanching may have helped with easy extractability by the formation of large ice crystals and breakdown of plant cell walls thereby aiding mass transfer of components. On the other hand, the significant loss of caffeic, coumeric, ferulic, hydroxybenzoic, rosmarinic and catechin in blanched mint may be either due to thermal degradation or loss of water soluble phenolics to surrounding blanching water. Furthermore, ferulic and rosmarinic acid without blanching seemed stable in freezing temperatures.

(a)



(b)



(c)

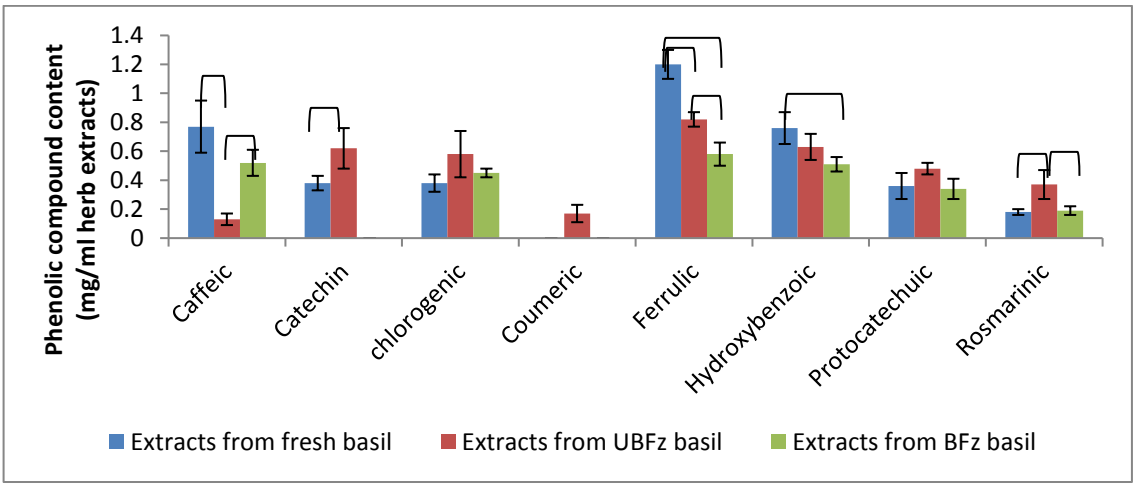


Figure 6.3 Plot of the representation of the HPLC determination of individual phenolic compounds of fresh, un-blanched frozen (UBF) and blanched frozen (Bf) (a) mint, (b) thyme and (c) basil extracts. Results are means of three readings of three different experiments. Values linked with ∩ are significantly different.

6.2.3 PREBIOTIC EFFECTS OF HERB EXTRACTS

Results of the prebiotic effects of aqueous extracts of analysed herbs showing different concentrations of herbs (10 and 20% v/v) are presented in Figures 6.5 and 6.6. The results varied among bacteria (*L.rhamnosus* and *B.bifidum*), herbs (mint, thyme and basil), treatments (fresh, un-blanched and blanched frozen) and concentration of extracts (10 and 20% v/v)

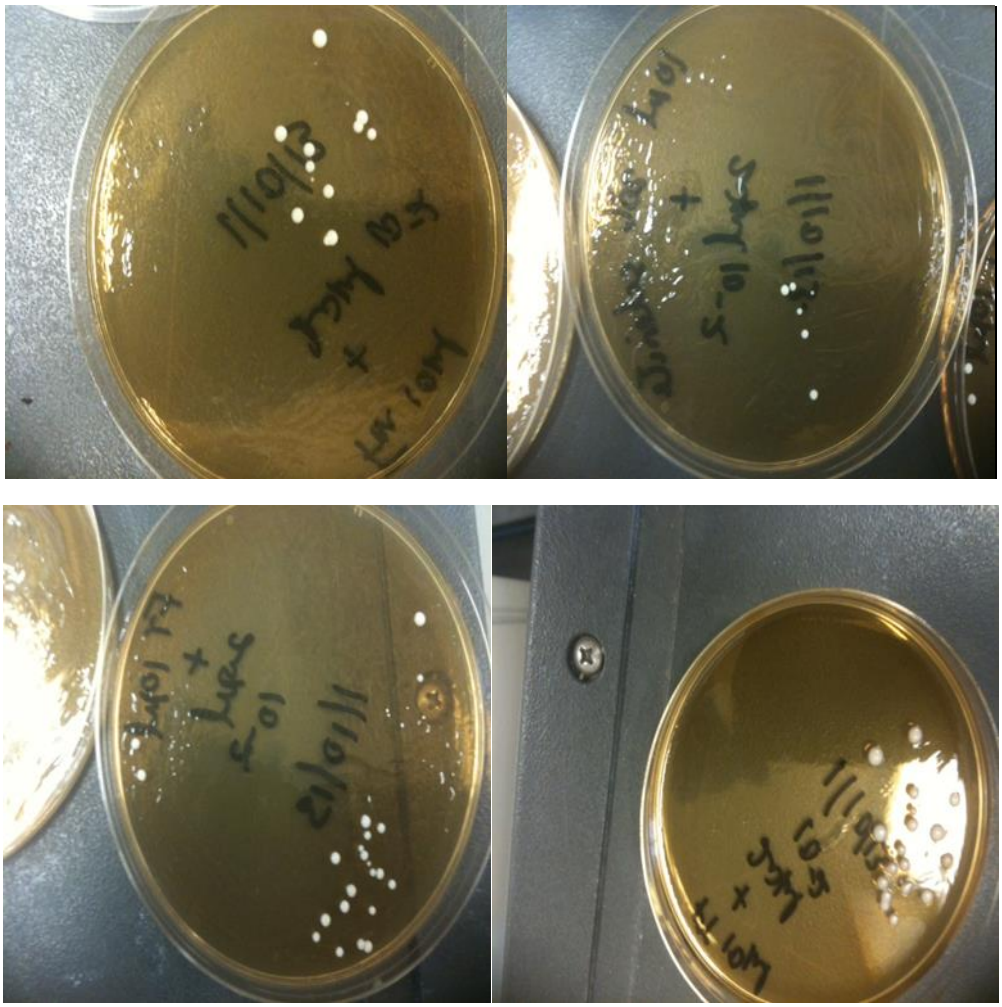


Figure 6.4 Picture representation of plates showing bacteria colony of *L.rhamnosus* and *B.bifidum* on nutrient agar

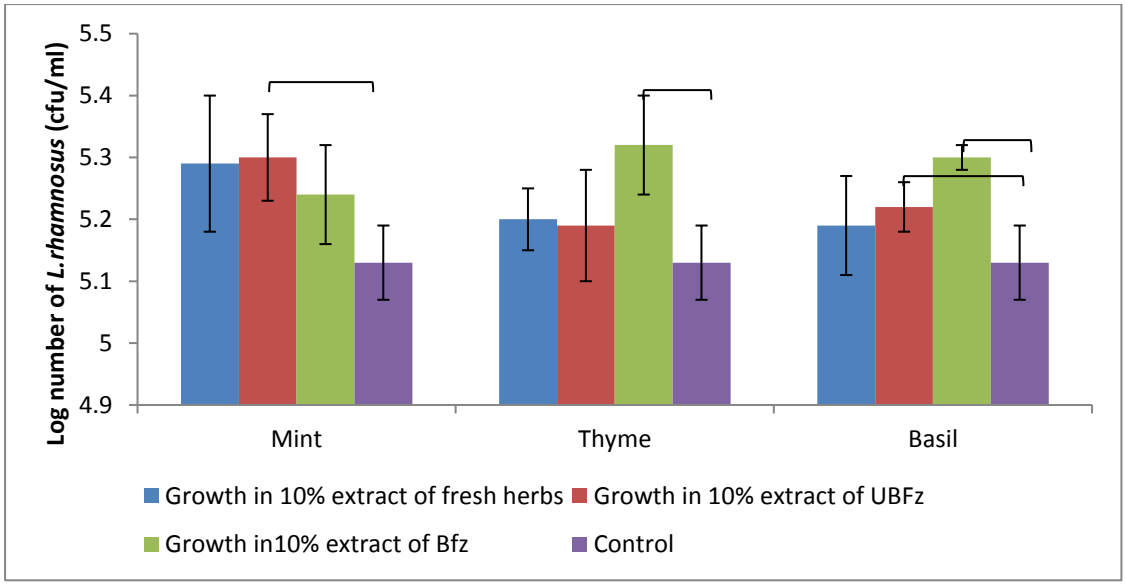
6.3.3.1 THE PREBIOTIC EFFECTS OF AQUEOUS HERB EXTRACTS ON *L.RHAMNOSUS*

From the results (Figure 6.5), addition of all extracts of fresh herbs led to slight but insignificant (P = 0.25) increase in bacteria cell growth of *L.rhamnosus* compared to control.

However, the addition of 10% of extracts from un-blanched frozen mint showed to have a significantly (P = 0.02) positive impact on the growth of *L.rhamnosus* cell compared to control (without extract), and extracts from fresh and blanched frozen mint. In addition, there is no significant difference (P = 0.52) between growths obtained with fresh and frozen (un-blanched and blanched) extracts. Furthermore, only extracts from blanched frozen thyme showed a significant (P = 0.02) increase in the log number of *L.rhamnosus* (cfu/ml) cell compared to extracts of fresh and un-blanched frozen thyme. However just like mint, there was no significant difference (P = 0.39) between observed growths in extracts of fresh and un-blanched frozen thyme.

Furthermore, with extracts from basil, apart from fresh extracts, all extracts from frozen (un-blanched and blanched frozen) basil showed significant increase in the log number of *L.rhamnosus* (cfu/ml) cell compared to control (P = 0.04 for un-blanched, and P = 2.22E-03 for blanched frozen samples). There was no significant difference between results obtained with extracts of fresh and frozen herbs (P = 0.59 for un-blanched, and P = 0.08 for blanched) but there was significant difference (P = 0.04) between results of extracts from un-blanched and blanched frozen basil.

(a)



(b)

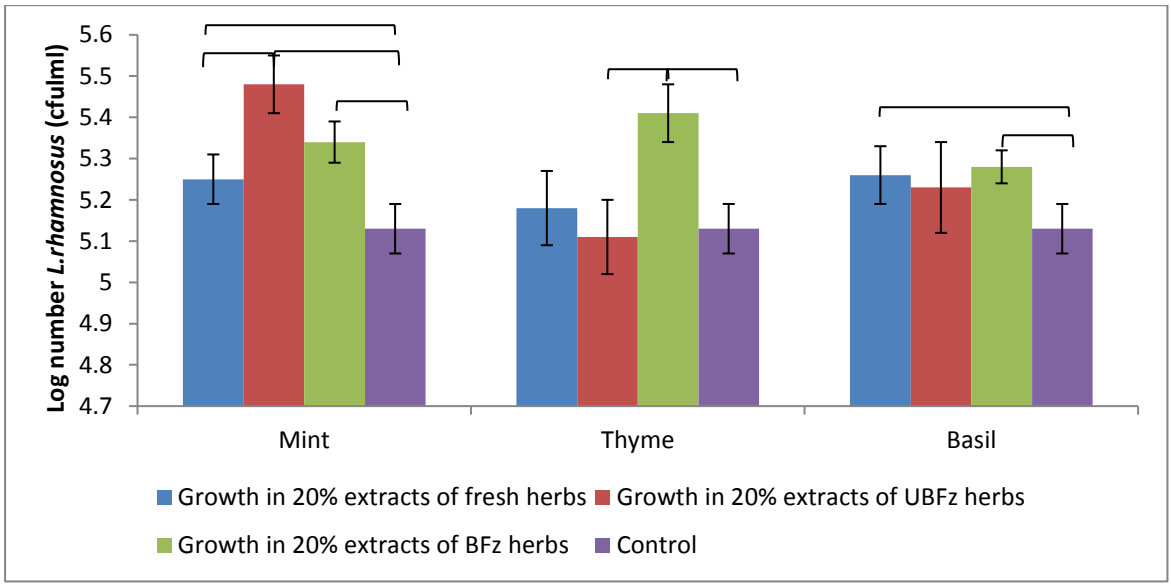


Figure 6.5 Plot of the representation of 10% v/v (a) and 20% v/v (b) of extracts from fresh, un-blanched (ubfz) and blanched frozen (bfz) herbs on the survival of *L.rhamnosus.* Results are means of three different extracts of independent herbs. Values linked with ∩ are significantly different (P < 0.05).

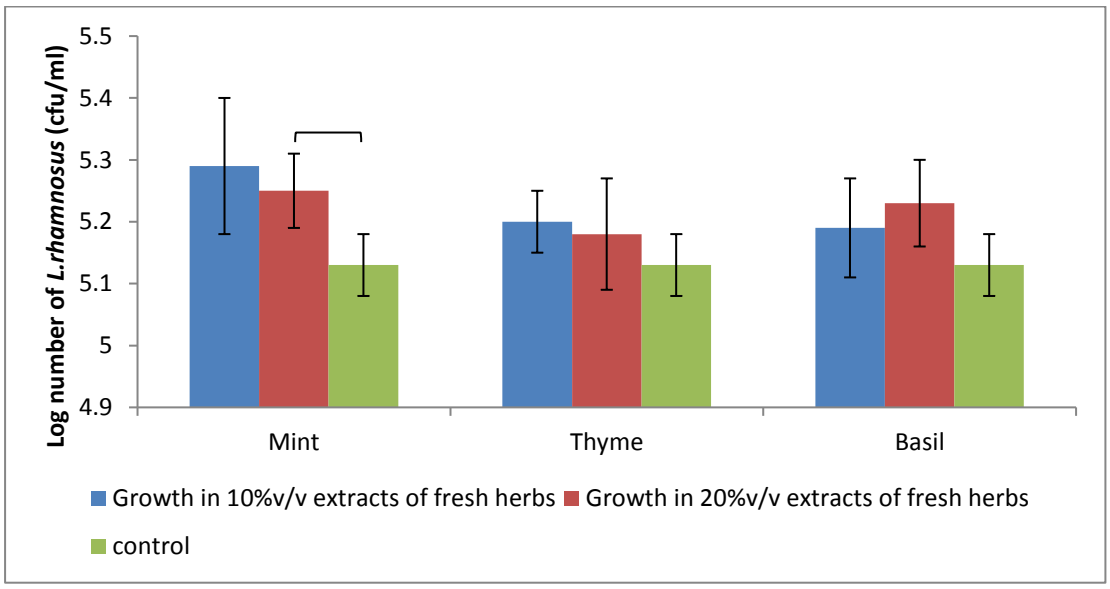
6.3.3.2 EFFECTS OF CONCENTRATION OF EXTRACTS ON THE GROWTH OF *L.RHAMNOSUS*.

The effects of increase in concentration of herb extracts on the log number of *L.rhamnosus* (cfu/ml) cell was tested and results are presented in Figures 6.6 a, b and c for fresh, un-blanched frozen and blanched from herbs respectively.

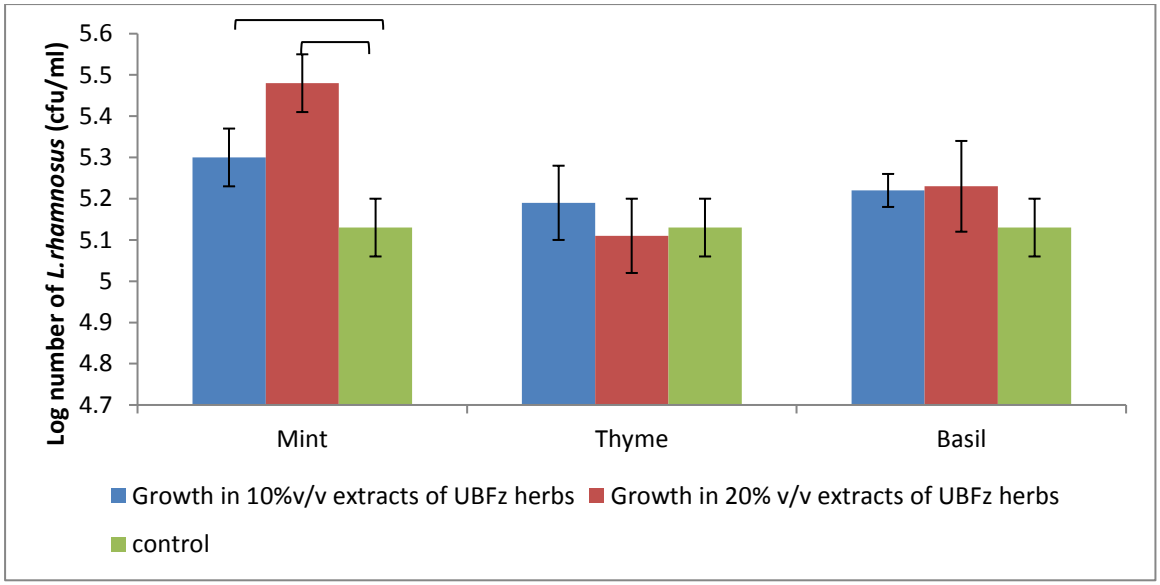
From these results, increase in concentration of mint extracts (10% to 20% v/v) led to a significant increase in the log number of *L.rhamnosus* (cfu/ml) cell compared to the control for fresh (P = 0.04), and frozen (P = 0.002 for un-blanched; P = 4.9E-03 for blanched) samples. However different results were observed with thyme and basil extracts. For thyme, only extracts from blanched frozen herb showed significant (P = 3.78E-03) increase in log number of *L.rhamnosus* (cfu/ml) compared to control. Furthermore, with basil both extracts from fresh and blanched frozen herbs showed significant increase in log number of *L.rhamnosus* (cfu/ml)

Generally apart from extracts of un-blanched frozen mint (Figure 6.6b) which showed a significant (P = 0.03) increase in the log number of *L.rhamnosus* (cfu/ml) cell with an increase in concentration of extracts (10% to 20% v/v), there was no significant difference (P = 0.61) with results obtained with increase in concentration of other herbs across treatments (fresh, un-blanched and blanched frozen). However slight to no increase in log number of *L.rhamnosus* (cfu/ml) cell was recorded with all herbs with increase in concentration (10% to 20% v/v) of herb extracts.

(a)



(b)



(c)

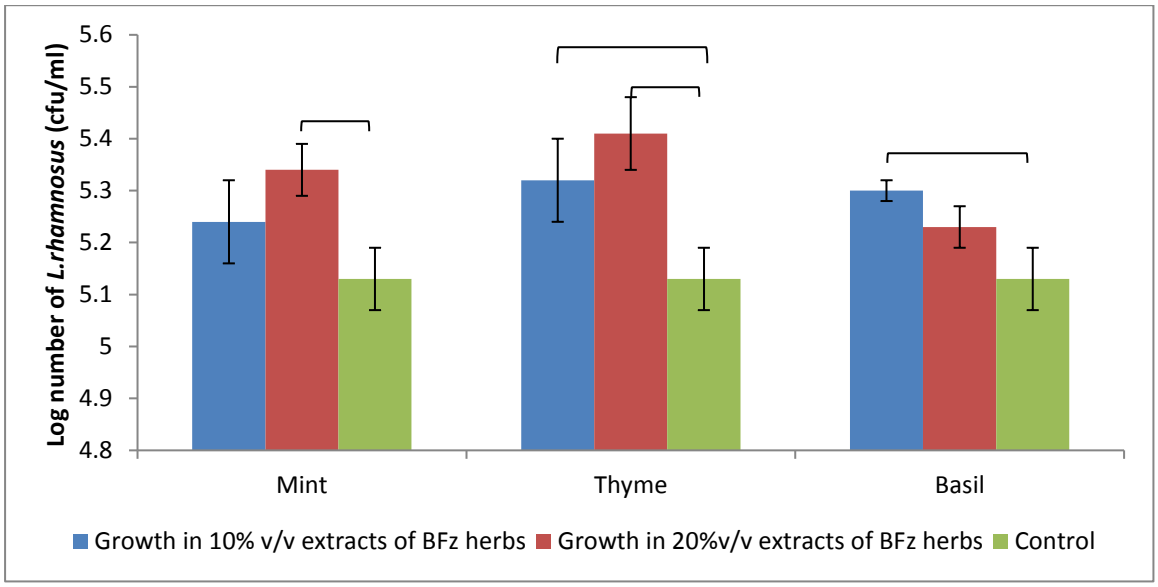


Figure 6.6 Plot showing effects of different concentrations (10% and 20% v/v) of water extracts of (a) fresh, (b) Un-blanched frozen (UBfz) and (c) Blanched frozen (Bfz) *Lamiaceae* herbs (Mint, thyme and basil) on the survival of *L.rhamnosus.* Results are means of three different extracts of independent herb. Values linked with ∩ are significantly different (P < 0.05).

6.3.3.3 CORRELATION BETWEEN PHENOLIC COMPOUDS AND SELENIUM CONTENTAND LOG NUMBER OF *L.RHAMNOSUS* (CFU/ML) CELL.

To find the relationship between phenolic compound, selenium and log number of *L.rhamnosus,* the correlation between the results of the prebiotic effects of different herb

extracts to their phenolic compounds and selenium content was determined. The correlations were shown to vary among treatments (fresh, un-blanched and blanched frozen).

The results showed strong positive correlation between caffeic acid, catechin, ferullic, hydroxybenzoic and rosmarinic acid content of extracts from fresh herbs and their prebiotic effects (R^2 = 0.65 to 0.98). Furthermore, a strong positive correlation also exists between the selenium content of extracts of fresh herbs and their prebiotic effects (R^2 = 0.94).

The correlations obtained with extracts of un-blanched frozen herbs were different. A strong positive correlation exists between prebiotic effects, coumeric, catechin, coumeric, ferrulic, hydroxybenzoic and rosmarinic acid (R^2 = 0.61 to 0.99).

Furthermore, results obtained extracts from blanched frozen herbs were totally different. The only positive correlation was obtained with chlorogenic acid (R^2 = 0.53).

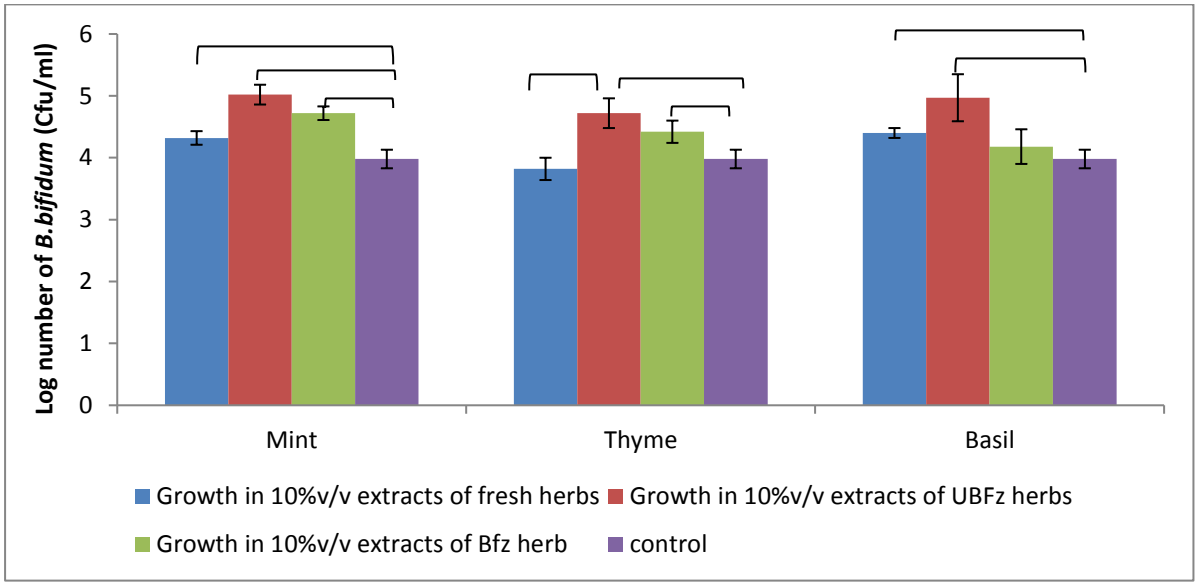
Although level of correlation varied across treatments (fresh, un-blanched and blanched frozen herbs), it can be concluded that the increase or decrease in log number of *L.rhamnosus* is as a result of the synergistic action of all phenolic compounds and selenium.

6.3.4 THE PREBIOTIC EFFECTS OF AQUEOUS HERB EXTRACTS ON *B.BIFIDUM*

Figure 6.7 shows the representation of the effects of herb extracts on the survival/growth of *B.bifidum*. From the results, addition of fresh herb extracts of both mint and basil led to a significant increase of *B.bifidum* compared to controls (P = 0.03 for mint, and P = 1.3E-02). However, there was a slight increase in the log *B.bifidum* cell (cfu/ml) but not significant (P = 0.3) increase in bacteria cells obtained with thyme extracts. Furthermore, all frozen herb extracts (un-blanched and blanched) gave significantly higher results compared to control and fresh herb extracts.

The results obtained from different herb extracts (mint, thyme and basil) were compared to one another. Generally, log *B.bifidum* cell (cfu/ml) in extracts from mint showed a higher result than other herbs (thyme and basil). However, between extracts of mint and thyme, apart from extracts from fresh herbs there was no significant difference (P = 0.42) between extracts from un-blanched frozen mint/thyme and blanched frozen mint/thyme.

(a)



(b)

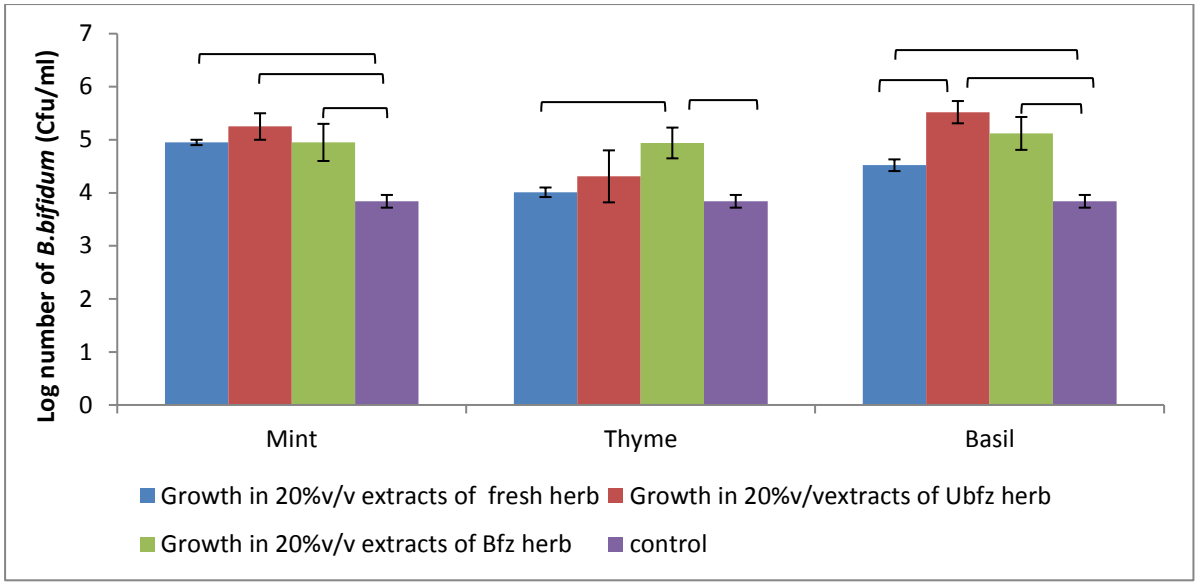


Figure 6.7 Plot of the representation of 10% v/v (a) and 20% v/v (b) of extracts from fresh, un-blanched (ubfz) and blanched frozen (bfz) herbs on the survival of *B.bifidum*. Results are means of three different extracts of independent herbs. Values linked with ∩ are significantly different (P < 0.05).

Figure 6.7b shows the representation of results of log of *B.bifidum* cell (cfu/ml) with a 20% (v/v) increase in the concentration of herb extracts. From the results, compared to control (without herb extracts), there is a significant (P = 1.22E-04) increase in the log of *B.bifidum* cell (cfu/ml) with a 20% (v/v) increase in the concentration of all mint extracts (fresh, un-blanched and blanched frozen). However, there was no significant difference (P = 0.29) between log of *B.bifidum* cell (cfu/ml) with a 20% (v/v) increase in the concentration between fresh and all frozen mint extracts. In contrast, with 10% (v/v) extract concentration (Figure 6.7a) there was significant difference (P = 2.41E-03) in log of *B.bifidum* cell (cfu/ml) between all treatments (fresh, un-blanched and blanched frozen). Generally, between results of log of *B.bifidum* cell (cfu/ml) obtained with 20% (v/v) increase of extracts of different treatments of mint, there was no significant difference between fresh and un-blanched frozen (P = 0.11); un-blanched and blanched frozen (P = 1).

Furthermore, compared with controls, there was no significant difference between increases in log of *B.bifidum* cell (cfu/ml) with a 20% (v/v) increase in the concentration of extracts from fresh and un-blanched frozen thyme (Figure 6.7b). This result is in contrast with those obtained with 10% (v/v) extracts of thyme (Figure 6.7a) where there was significant difference between the log of *B.bifidum* cell (cfu/ml) of extracts of fresh and un-blanched frozen thyme. The only

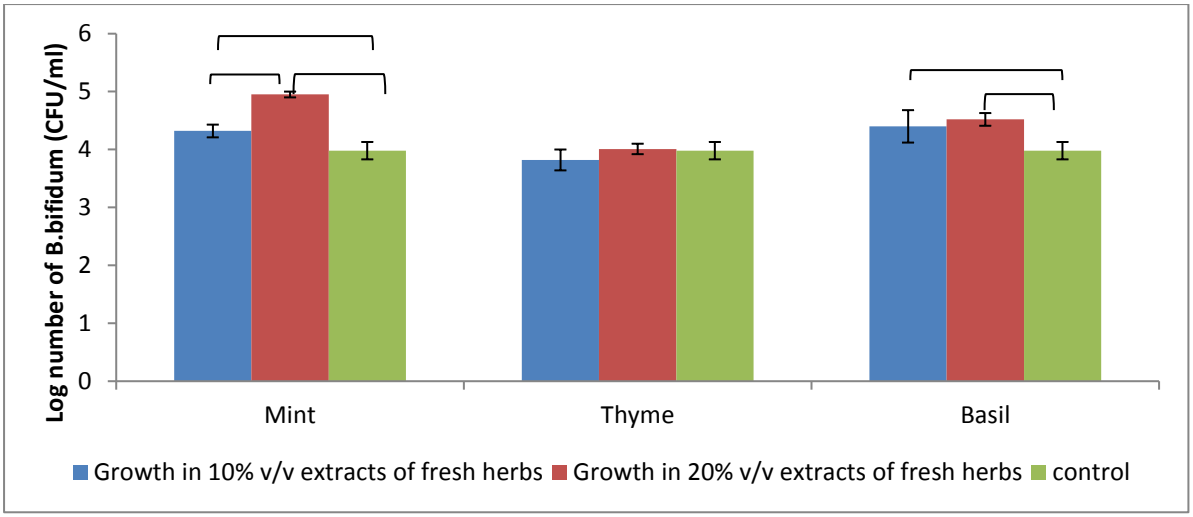
significant difference (P = 6.1E-03) exists between extracts from fresh and blanched frozen thyme.

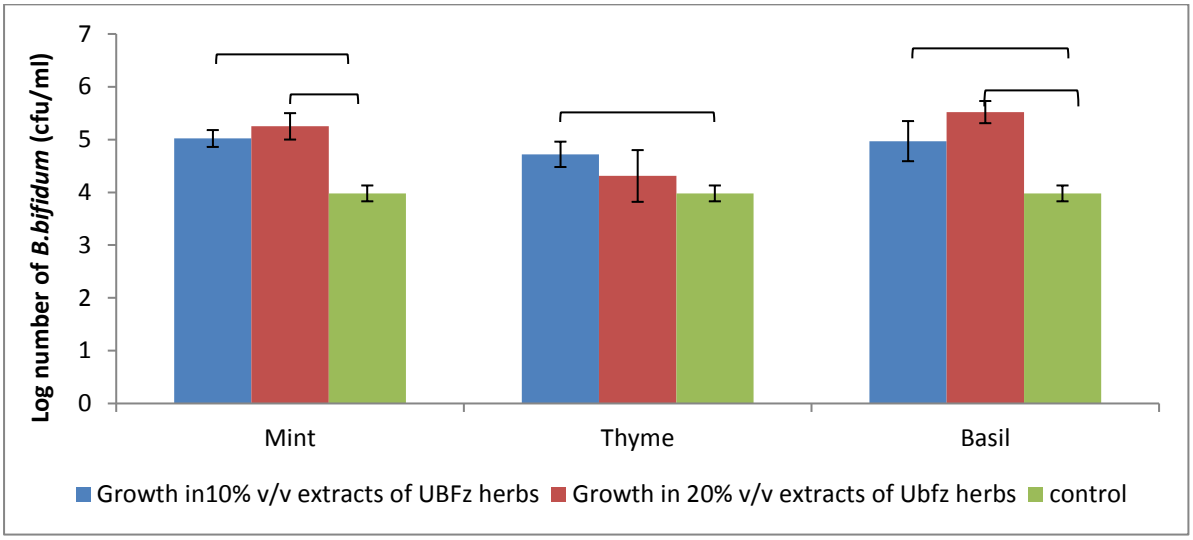
In contrast with results obtained with thyme, there was a significant increase in log of *B.bifidum* cell (cfu/ml) with a 20% (v/v) increase in the concentration of extracts of fresh (P = 1.94), un-blanched (2.74E-04), and blanched frozen (P = 2.6E-03) basil compared to control. In contrast, with 10% extract concentration (Figure 6.7a) there was a significant difference (P = 2.6E-03) between log of *B.bifidum* cell (cfu/ml) of control and extracts from blanched frozen basil. Furthermore, there was significant difference between log of *B.bifidum* cell (cfu/ml) of in 20% (v/v) extracts from fresh and all frozen basil but there was no significant difference (P = 0.13) between log of *B.bifidum* cell (cfu/ml) in extracts from un-blanched and blanched frozen basil.

6.3.4.1 EFFECTS OF CONCENTRATION OF EXTRACTS ON THE GROWTH OF *B.BIFIDUM*.

Concentration of herb extracts was increased to test if growth of bacteria cells are concentration dependant and results are presented in Figures 6.8 a, b and c for extracts from fresh, un-blanched and blanched frozen herbs respectively.

From the results, compared to 10% (v/v) of extracts, an increase in concentration of extracts from fresh mint (20% v/v) led to a significant (P = 8.3E-04) increase in the log of *B.bifidum* (cfu/ml) cell (Figure 6.8a). In contrast, there was no significant increase in the growth with an increase in concentration of all extracts (20% v/v) of thyme (P = 0.18 for fresh, P = 0.26 for un-blanched, P = 0.06 for blanched frozen) compared to 10% (v/v). Furthermore, with basil, there was no significant difference in the log of *B.bifidum* (cfu/ml) cell with increase in concentration of extracts (20% v/v) from fresh (P = 0.2) and un-blanched frozen (P = 0.09) basil (Figure 6.8a and b respectively) compared to 10% (v/v) of extracts. However, a significant difference (P = 0.02) in the increase in the log of *B.bifidum* (cfu/ml) cell was recorded with increase (20% v/v) in the concentration of extracts from blanched frozen basil (Figure 6.8c) compared to 10% (v/v) of extracts.


Log number of B.bifidum (CFU/ml)
0
1
2
3
4
5
6
Mint
Thyme
Basil
Growth in 10% v/v extracts of fresh herbs
Growth in 20% v/v extracts of fresh herbs
control


Log number of B.bifidum (cfu/ml)
0
1
2
3
4
5
6
7
Mint
Thyme
Basil
Growth in10% v/v extracts of UBFz herbs
Growth in 20% v/v extracts of Ubfz herbs
control

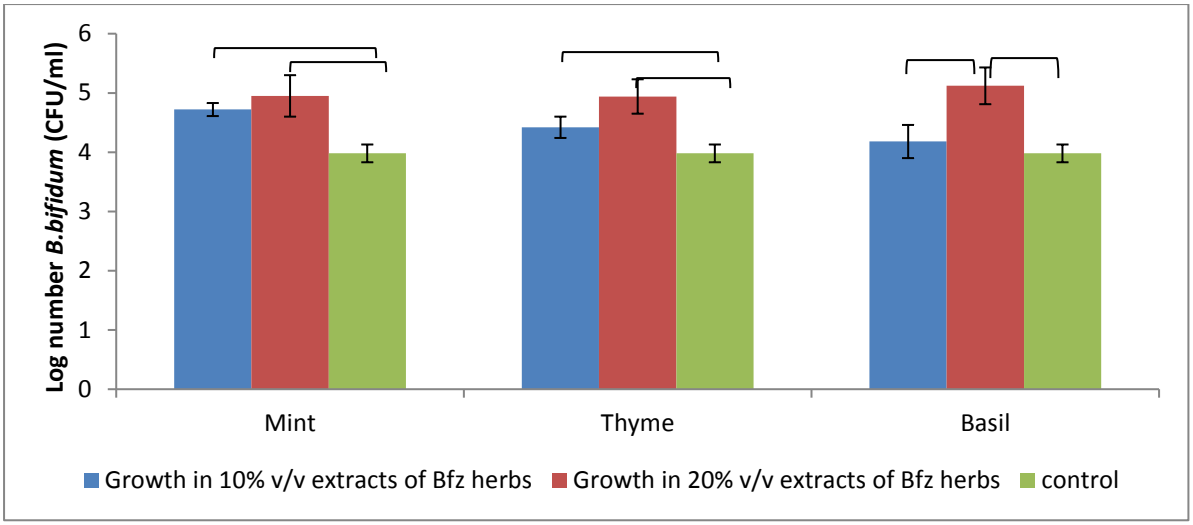


Figure 6.8 Plot showing effects of different concentrations (10% and 20% v/v) of water extracts of (a) fresh, (b) Un-blanched frozen (UBfz) and (c) Blanched frozen (Bfz) *Lamiaceae* herbs (Mint, thyme and basil) on the survival of *B.bifidum.* Results are means (± SD) of three different extracts of independent herbs. Values linked with ∩ are significantly different (P < 0.05).

6.3.4.2 CORRELATION BETWEEN SELENIUM, DIFFERENT PHENOLIC COMPOUND CONTENT OF HERB EXTRACTS AND LOG NUMBER OF *B.BIFIDUM* (CFU/ML) CELL.

Correlations between phenolic compound content of extracts and their prebiotic effect on the log number *B.bifidum* (cfu/ml) cell is determined using a Pearson's correlation matrix.

A high positive correlation was obtained between the prebiotic effects of extracts from fresh herbs and caffeic acid (R^2 = 0.92), chlorogenic acid (R^2 = 0.90), ferrulic acid (R^2 = 0.79) and hydroxybenzoic acid (R^2 = 0.65) which increased with increase in extract concentration. These maybe as a result of fairly high concentration of these phenolic compounds in extracts of fresh herbs. Food products with high concentration hydrocinnamates such as caffeic acid, p-coumeric acid and ferrulic acid have been reported as to have positive prebiotic properties to probiotic bacteria (Guglielmetti *et al*., 2008).

For extracts from un-blanched frozen herbs, fairly high positive correlation exists between prebiotic effects on the log number of *B.bifidum* (cfu/ml) cell and caffeic acid (R^2 = 0.65), catechin (R^2 = 0.61), chlorogenic acid (R^2 = 0.60), ferrulic acid (R^2 = 0.82), rosmarinic acid acid (R^2 = 0.69). The highest positive correlation was obtained with protocatechuic acid (R^2 = 0.98).However an increase in concentration of herb extracts (20% v/v) only led to an increase

in correlation between log number of *B.bifidum* (cfu/ml) cell and caffeic acid (R^2 = 0.88), protocatechuic acid (R^2 = 0.98) and chlorogenic acid (R^2 = 0.85).

Results of correlation between log number of *B.bifidum* (cfu/ml) cell and phenolic compounds of extracts from blanched frozen herbs showed a high positive correlation with catechin (R^2 = 0.99), coumeric acid (R^2 = 0.90), rosmarinic acid (R^2 = 0.82), ferrulic acid (R^2 = 0.83), hydroxybenzoic acid (R^2 = 0.62) and protocatechuic acid (R^2 = 0.44). However irrespective of a significantly higher (P = 2.03E-04) in chlorogenic acid content of extracts from blanched frozen herbs, a very low correlation was obtained between log number of *B.bifidum* (cfu/ml) cell and chlorogenic acid content. Chlorogenic acid which is an ester of caffeic acid and quinic acid has been reported to be more active if hydrolysed by cinnamoyl esterase to free caffeic acid which has a more active prebiotic property (Rossi and Amaretti, 2010).

6.4 DISCUSSIONS

The principal finding of this study is that aqueous extracts of common culinary herbs of *lamiaceae* family (mint, thyme and basil) are effective at promoting the growth of pure cultures of *L.rhamnosus* and *B.bifidum*. The herb extracts also possessed high total phenolic content and antioxidant activity determined as radical scavenging ability (%) with high phenolic compound content, which varied across herbs and treatments.

Results showed that there is no significant difference (P > 0.05) between the growth effects on *L.rhamnosus* caused by different herbs irrespective of treatment (fresh or frozen). However, for *B.bifidum* the growth effects of herbs varied among herbs with some treatments. For instance, there is a significant difference between the growths of *B.bifidum* in extracts of fresh mint and thyme; extracts of fresh thyme and basil; extracts of blanched frozen thyme and basil.

To some extent, the hypothesis that selenium and some phenolic compounds may have effect on growth of probiotic bacteria is proven.

Generally there was high positive correlation between selenium content of all extracts of herbs (fresh, un-blanched frozen and blanched frozen herbs) and the growth of *L.rhamnosus* and *B.bifidum*. This implies that the selenium content of herbs may be a great contributor to the increase in the log number of probiotic bacteria cells. Previous reports have shown that high selenium content significantly enhances bacteria growth (Aruz *et al*., 2007, Molan *et al*., 2009).

Although other health promoting effects of these herbs have been reported, no research has been carried out so far on their effects as prebiotics to probiotic bacteria. However, there have been few studies which have shown that green tea and its polyphenols can promote the growth of beneficial probiotic bacteria (Goto *et al.,* 1998; Hara *et al.,* 1995; Ishihara et al., 2001; Molan *et al*., 2009). Furthermore, using certain apple cultivars and tea infusion in the presence of skimmed milk, Guglielmetti *et al.* (2007) developed a functional food product which promoted the growth of some lactic acid bacteria. The authors likened these properties to the presence of phenolic compounds such as hydrocinnamates (Yuan et al., 2005; Guglielmetti *et al.* 2007), catechin (Goto *et al.,* 1998; Hara *et al.,* 1995; Ishihara et al., 2001; Molan *et al*., 2009) and selenium (Molan et al., 2009).

The mechanism by which these phenolic compounds contributed towards increased the growth of *L.rhamnosus* and *B.bifidum* in this study is not clearly known. However, a close explanation may be that apart from their various health promoting functions (Suzuki *et al*., 2002; Tanaka *et al*., 1993; Takeda *et al*., 2002), phenolic compounds, especially free hydrocinnamates (caffeic, ferrulic and p-coumeric acids) are known to act as carbon source to probiotic bacteria (Yuan *et al*., 2005). Furthermore, due to their antioxidative functions, phenolic compounds are said to modulate the oxidative stress in medium generated by metabolic activities and consequently provide a better environment for the growth and multiplication of these bacteria cells (Molan *et al*., 2009). The growth of probiotic bacteria may also be as a result of a symbiotic action between bacteria strains, phenolic compounds or other un-assayed phytochemicals of chosen herb origin. Some research has reported the ability of several strains of *Bifidobacteria* to hydrolyse glycosilated forms of some phytochemicals, including flavonoids and glucosinolates, into more bioactive compounds (Cheng *et al*., 2004; Raimondi *et al.,* 2009).

Furthermore, Shetty (2004) proposed that phenolic antioxidants do not only act as direct ROS scavengers but also acts to stimulate certain enzymatic pathways in cells. One of such pathways is the pentose phosphate pathway which involves oxidation of glucose and supplies metabolic intermediate for biosynthetic processes.

6.5 CONCLUSIONS

Extracts of selected herbs can be seen to be novel functional ingredient for the development of functional food products which exhibit high phenolic compound content, antioxidant power and at the same time presenting prebiotic effects to two popular autochontiuos probiotic bacteria. The issue of eliminating/discouraging the growth/proliferation of pathogenic bacteria, *lamiaceae* herbs, especially *mentha piperita* (mint) and their respective HPLC identified phenolic compounds in extracts such as rosmarinic acid and caffeic acid are well known and have been widely reported as phenolic compounds with strong antimicrobial effects (Bupesh *et al.*, 2007).

This study utilised an *in vitro* assay with several assumptions linking other phenolic compounds such as rosmarinic acid, protocatechuic and hydroxybenzoic acid with probiotic growth utilizing correlation coefficients, further work needs to be carried out on each phenolic compound and their prebiotic effect. Although works have been carried out on hydrocinnamtes, selenium and tea catechin, no work has been carried out on phenolic compounds such as rosmarinic acid and the hydroxybenzoic acids which at some point showed high correlation with probiotic bacteria growth.

Finally, freezing (blanched or un-blanched) did not have significant effects on the growth of *L.rhamnosus* irrespective of herbs while significant effect was observed on the growth of *B.bifidum* with thyme and basil.

CHAPTER 7

GENERAL DISCUSSION

The main objective of this research was to investigate the extent to which blanching and freezing affect some functional constituents and properties of three *lamaiceae* herbs (*Mentha Piperata, Thymus Vulgaris* and *Ocimuim basilica*). The constituents investigated are mainly antioxidative compounds which are deemed as the important constituents for the cellular defence system. These include phenolic compounds, ascorbic acid (total, oxidized and reduced ascorbic acid), selenium and phytic acid. Furthermore, some of them, especially ascorbic acid been susceptible to heat damage/degradation, are a measure of the effects of processing on food stuffs.

The functional properties investigated include the antioxidant activity, enzyme inhibition ability (control of postprandial blood glucose level and hypertension) and prebiotic functions.

Generally, mint (*Mentha Piperita*) showed the highest and the most superior functional constituent content and properties among all other assayed herbs irrespective of treatment (blanched or frozen) given.

Results of the total antioxidant activity determined as DPPH inhibition ability, FRAP, ORAC and CUPRAC varied among extracts and extracting solvents. This depended on the antioxidant compounds extractible in individual solvents (water and methanol) and their respective antioxidant activity. Furthermore, freezing and blanching have shown either to increase or decrease in the value of total antioxidant activities. Several inconsistent reports have also been given on the effects of freezing and blanching on some antioxidant activity of some other vegetables and plants (Chan, et al, 2014; Kim et al, 2013; Pujimulyani, et al, 2012, Wen *et al*, 2010, Yamaguchi et al, 2001). These reports vary from increased, decreased to insignificant effects. For instance, Kim, et al (2013) reported a significant reduction in ferric reducing property, with no significant alteration of the DPPH and ABTS scavenging activity of blanched caster aralia and dureup compared to fresh products. Furthermore, research by Chan, et al. (2014), reported that blanching of anacardium occidentale (cashew), cosmos caudatus (wild cosmos), polygonum minus (smart weed) and piper betle (betel) led to a significant loss in phenolic content and antioxidant activity, measured as total phenolic content (TPC), total flavonoid content (TFC), caffeoylquinic acid content (CQAC), ascorbic acid equivalent antioxidant capacity, and ferric reducing power. In contrast, blanching led to the increase in

ferrous reducing capacity of asparagus, burdock, carrot, eggplant, green chilli (Yamaguchi et al, 2001), and rhizomes of saffron (Pujimulyani, et al, 2012). Furthermore, Pujimulyani, 2012 reported that HPLC analysis of blanched and fresh saffron showed significant increase in quercetin in blanched rather than in fresh and linked it to hydrolysation of one of the identified glycoside compounds - quercetin-3-rutinoside. Furthermore, on the influence of freezing (-18°C), Oluai et al (2015), reported that during this process there was no significant loss of the antioxidant capacity (DPPH scavenging ability) and total phenolic contents of five leafy vegetables (*Hibiscus sabdariffa, Amaranthus hybridus, Andasonia digitata, Vigna unguiculata and Ceiba pentandra*) after 1 month of freezing. However, there was significant loss of 2.06 – 19.67% after prolonged freezing period of 2 and 3 months. This coincides with the report of Mullen et al (2002), who found no significant difference between antioxidant capacities of frozen and fresh Scottish raspberries after short freezing period of 24hrs, and a significant loss after prolonged (12months) frozen storage.

On the effect of blanching/heat treatment and freezing on selenium, content of herbs, this research reported an increase in the selenium content of herbs. Although there are no reports on the effects of freezing and blanching on selenium content of herbs or other plant products, Mo, et al, (2006) observed significant loss of selenium content of cabbage during blanching. These all indicate that pretreatment can have a significant effect on the preservation of selenium in selenium enriched products.

Furthermore, in this research, results of phytic acid content of herbs clearly showed a significant loss in the phytic acid content of samples when frozen (un-blanched and blanched). Although no research has reported on the effects of freezing on the phytic acid content of food substances, several researches have reported on the effects of blanching on the phytic acid content of plant products. These include, the loss of phytate on blanching of *Moriinga Oleifere* by 39.8% (Salau, et al, 2012), and the same effect in some non-conventional African vegetables (Nakafamiya, et al, 2010). Furthermore, Helbig, et al (2003), reported that freezdrying reduced the phytic acid content of common beans (*Phaseolus vulgaris, L.*) to 60.8% compared to soaking in water (20.9%).

Generally, results of the total antioxidant activity clearly indicate that freezing (blanched or unblanched) of assayed herbs alters their antioxidant activity. This also varies with individual herbs and the extraction solvent. However, based on results obtained and their analysis,

methanol extracts were generally shown to contain more antioxidant compounds that possess DPPH, FRAP, CUPRAC, and ORAC ability. Hence for herbal infusion, methanol proved to be a better extraction solvent.

Results of the key carbohydrate hydrolyzing enzyme inhibition ability of herbs generally indicates that assayed herbs can be used for the control of postprandial blood glucose level after a carbohydrate meal. Furthermore, a desired moderate/low porcine pancreatic α-amylase (PPA) was obtained. Low to moderate PPA is said to be desired due to the adverse effects of high inhibition obtained by the drug acarbose (Nikavar *et al*, 2008; Wongsa *et al*, 2012; Sudha *et al* 2011). However, no research has reported the effects of freezing treatments (blanched/unblanched) on the α-amylase inhibition activity of herbs.

Results have shown that the highest inhibition was obtained by fresh herbs followed by unblanched frozen herbs with the blanched frozen herbs giving the lowest PPA inhibitory activity. One major drawback of past reports on α-amylase inhibitory activity and the adverse effects caused by over/high inbitory activity by the drug acarbos is that there are no figures indicating any definite or range of safe level of α-amylase. Since this research utilized an *in vitro* PPA inhibition assay, what is a low/moderate level may be too low or too high to be regarded as safe α-amylase inhibition activity. Hence, for further studies, the use of human intervention studies to set definit range (in percent) to show safe or over inhibitory activity for enzyme α-amylase may be warranted.

Results of α-glucosidase inhibition activity show high inhibitory activity of all herbs. However, mint showed the highest ability across all treatments which decreased on freezing with blanched frozen herbs showing the least inhibition activity. Freezing enhanced the α-glucosidase inhibitory activity of both thyme and basil which was not obtained with fresh herbs. In addition to affecting the level of inhibition of α-glucosidase, results of enzyme kinetics also indicate that extracts of fresh and unblanched herb showed un-competitive mode of inhibition while blanched frozen herbs showed mixed inhibition. Furthermore, unblanched frozen herbs showed the strongest inhibitor with the lowest IC_{50} followed by fresh and blanched frozen as the least. Therefore, freezing (blanching/ unblanched) can be used to alter the enzyme kinetics/mode of inhibition activity and the inhibition strength of herbs.

No research has reported on the control of postprandial blood glucose level after a carbohydrate meal by these enzyme inhibitions or the effects of freezing using fresh herbs. Hence for future research, in addition to the key carbohydrate hydrolyzing enyme inhibition activity of herbs and other food products, all forms (fresh, dried, frozen) should be used to see if the inhibition activity/mode can be enhanced/altered.

Based on the findings of the α-glucosidase inhibition activity of herbs, it can be concluded that if using mint for α-glucosidase inhibition activity, better results/higher inhibition effects will be obtained using fresh mint rather than frozen or blanched mint. Furthermore, to achieve a stronger inhibition effect with mint, it is advisable to use un-blanched frozen samples since it showed a significantly low IC_{50} compared to both fresh, and blanched frozen mint. Furthermore, considering the results obtained with thyme and basil, better α-glucosidase inhibition activity will be obtained if thyme and basil are froen (blanched or un-blanched).

The principal finding of the prebiotic effects of herb extracts is that all herbs irrespective of treatment (fresh or frozen) showed significant prebiotic effects on *L.rhamnosus* and *B.bifidum*. However, freezing (blanched or unblanched) didn't seem to significantly affect the growth of *L.rhamnosus* while significant effect was obtained with *B.bifidum* using frozen (blanched or unblanched) extracts of thyme and basil. These results are attributed to phenolic compounds which have shown high correlation with the results of prebiotic effects. Phenolic compounds have in past studies been reported to have positive effects on the growth of some lactic acid bacteria (Isihara *et al*, 2001; Molan *et al*, 2009). One of the very few explanations given to this is that phenolic compounds, especially free hydrocinnamates (caffeic, ferrulic and p-coumeric acids), are known to act as a carbon source to probiotic bacteria (Yuan *et al*, 2005). However, the mechanism of action of these phenolic compounds on probiotic bacteria has not been properly elucidated. Hence for future work, the use of appropriate analytical method/tools should be utilized to ascertain the meachanism of action of the phenolic compounds of the assayed herbs. Furthermore, using human intervention studies, research should be carried out on the transition and assimilation of extracts of these herbs through the human colon to execute its prebiotic function.

Finally, based on the results of the prebiotic effects of herbs on probiotic bacteria *L.rhamnosus* (fresh, blanched and unblanched frozen), it can be concluded that all herbs, irrespective of

treatment (fresh, blanched and unblanched frozen), give similar effects. Hence, level of prebiotic effect of fresh herbs is similar to that obtainable if herbs are fozen (blanched or unblanched). However, there was a significant difference between the effects of herbs on the growth of *B.bifidum* , especially with extracts of basil; with better responses obtained when mint, thyme and basil are frozen (un-blanched and blanched) samples.

In conclusion, with variations in results across herbs and treatment, freezing and blanching prior to freezing is a possible tool for the manipulation of the assayed functional constituents and properties of mint, thyme and basil.

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APPENDIX

NOTE:

DPPH - Diphenyl picryl hydrazl assay inhibition assay (% inhibition)

FRAP –Ferrous reducing antioxidant potency (µmol Fe II equivalent/L)

CUPRAC – Copper reducing antioxidant reducing activity (mg trolox equivalent/ g dw of herb)

ORAC- Oxygen radical absorption capacity (mg trolox equivalent/g dw of herb)

Total phenolic content – mg gallic acid equivalent/g dw herb)

Herb	Moisture loss (%)	Extraction solvent	Total phenolic content	Antioxidant activity			
				DPPH	FRAP	CUPRAC	ORAC
Flat leaf parsley	80.00 ± 5.77	Water	1.35 ± 0.06	3.36 ± 1.32	56.61 ± 13.50	38.33 ± 2.01	92.11 ± 10.10
		Methanol	64.68 ± 7.61	6.49 ± 4.07	259.46 ± 34.31	173.75 ± 18.17	52.66 ± 11.93
		RMCD	30.44 ± 3.51	8.84 ± 1.65	213.36 ± 20.13	157.15 ± 12.77	51.88 ± 6.54
		PBS	15.32 ± 1.72	ND	172.45 ± 42.48	86.09 ± 12.20	89.20 ± 21.84
Curly leaf parsley	81.88 ± 2.04	Water	1.11 ± 0.17	1.06 ± 0.72	44.29 ± 20.14	13.09 ± 1.50	75.44 ± 11.46
		Methanol	47.33 ± 15.18	7.50 ± 2.49	216.33 ± 16.49	147.94 ± 12.71	76.68 ± 6.74
		RMCD	22.18 ± 1.92	6.67 ± 0.54	201.55 ± 47.58	174.32 ± 10.81	57.80 ± 20.83
		PBS	8.91 ± 0.99	ND	171.55 ± 22.87	63.58 ±18.34	39.42 ± 3.40
Corianda	86.88 ± 0.62	Water	8.35 ± 1.08	37.26 ± 12.36	315.36 ± 71.81	54.98 ± 7.11	43.58 ± 11.68
		Methanol	72.33 ±10.62	23.14 ± 14.09	543.21 ± 58.89	199.52 ±25.83	77.40 ± 12.94
		RMCD	50.19 ± 13.67	9.97 ± 0.54	359.73 ± 60.74	198.13 ± 21.78	23.13 ± 9.79
		PBS	21.43 ± 1.85	ND	261.55 ± 17.75	106.55 ± 9.28	47.26 ± 13.80
Mint	85.00 ± 1.96	Water	37.98 ± 3.25	79.58 ± 10.72	672.69 ± 66.32	54.82 ± 9.24	44.12 ± 9.06
		Methanol	284.20 ± 50.25	85.62 ± 5.25	3828.10 ± 304.16	273.97 ± 53.77	111.28 ± 29.49
		RMCD	115.77 ± 10.25	22.71 ± 2.01	1533.63 ± 210.78	393.10 ± 85.45	148.00 ± 15.93
		PBS	47.95 ± 5.33	ND	1824.55 ± 152.08	87.12 ± 20.07	15.34 ± 2.70
Thyme	89.38 ± 0.23	Water	12.61 ± 0.97	35.32 ± 5.74	348.65 ± 40.79	9.14 ± 0.98	40.71 ± 10.62
		Methanol	87.16 ± 14.19	86.42 ± 9.50	3373.35 ± 431.93	167.78 ± 36.05	56.28 ± 18.24
		RMCD	62.02 ± 5.03	19.84 ± 0.44	1037.00 ± 42.35	313.45 ± 14.57	110.93 ± 41.85
		PBS	38.77 ± 0.52	ND	1633.64 ± 121.17	64.95 ± 3.04	10.28 ± 2.10
Basil	90.63 ± 5.61	Water	4.66 ± 0.54	13.36 ± 1.95	149.55 ± 31.87	20.78 ± 3.07	75.11 ± 10.57
		Methanol	98.09 ± 20.02	87.84 ± 6.70	2541.39 ± 230.71	343.09 ± 31.05	81.43 ± 12.56
		RMCD	40.16 ± 0.99	7.54 ± 3.02	1215.45 ± 131.08	448.52 ± 34.57	76.54 ± 10.73
		PBS	41.55 ± 6.78	ND	1779.09 ± 221.74	106.92 ± 41.24	72.50 ± 11.90
Chive	91.88 ± 2.29	Water	2.56 ± 0.77	0.44 ±0.32	24.93 ± 11.89	52.63 ± 11.09	172.13 ±26.42
		Methanol	44.14 ± 10.02	7.94 ± 2.46	256.86 ± 37.06	194.77 ± 19.88	81.00 ± 11.51
		RMCD	15.54 ± 2.41	12.05 ± 1.59	245.18 ± 11.71	290.43 ± 46.57	268.21 ± 60.41
		PBS	5.28 ± 0.25	ND	187.00 ± 33.48	147.36 ± 15.02	124.53 ± 23.08

Table 1. TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY ASSAY OF FRESH HERBS

Herb	Moisture loss (%)	Extraction solvent	Total phenolic content	Antioxidant activity			
				DPPH	FRAP	CUPRAC	ORAC
Flat leaf parsley	85.32 ± 5.19	Water	1.22 ± 0.74	1.26 ± 0.55	33.17 ± 3.19	22.12 ± 5.31	26.97 ± 7.05
		Methanol	45.34 ± 2.56	2.03 ± 0.80	208.01 ± 9.02	150.11 ± 7.05	118.03 ± 18.11
		RMCD	12.01 ± 1.72	6.41 ± 0.32	1393.23 ± 110.42	219.17 ± 16.04	51.00 ± 4.13
		PBS	26.11 ± 4.09	ND	158.11 ± 13.04	74.71 ± 11.09	21.93 ±2.16
Curly leaf parsley	85.10 ± 0.99	Water	1.85 ± 0.48	1.03 ± 0.05	32.21 ± 7.13	9.15 ± 1.05	44.73 ± 3.62
		Methanol	38.13 ± 5.02	1.99 ± 0.63	188.29 ± 11.52	113.21 ± 5.12	126.16 ± 5.54
		RMCD	15.11 ± 1.52	6.73 ± 1.47	1402.07 ± 112.26	162.10 ± 8.43	63.02 ± 4.12
		PBS	9.34 ± 0.76	ND	160.35 ± 4.73	57.33 ± 7.61	33.42 ± 1.19
Corianda	82.71 ± 7.21	Water	5.99 ± 1.05	17.78 ± 2.03	200.00 ± 21.18	10.21 ± 1.82	80.73 ± 10.03
		Methanol	51.11 ± 4.07	11.12 ± 1.58	233.38 ± 17.03	139.99 ± 17.02	135.41 ± 7.10
		RMCD	35.70 ± 2.17	7.23 ± 1.21	1401.42 ± 31.09	185.78 ± 9.08	28.14 ± 9.07
		PBS	26.18 ± 3.32	ND	229.23 ± 12.41	125.03 ± 12.03	131.16 ± 2.08
Mint	86.69 ± 4.41	Water	36.77 ± 7.92	93.91 ± 4.09	1413.10 ± 42.23	7.11 ± 2.20	161.05 ± 3.61
		Methanol	222.17 ± 16.02	88.58 ± 3.18	2392.06 ± 88.76	155.74 ± 8.19	94.80 ± 7.53
		RMCD	90.95 ± 11.02	10.72 ± 0.35	1922.11 ± 9.43	233.13 ± 24.01	30.02 ± 6.21
		PBS	48.53 ± 9.81	ND	1631.64 ± 33.61	114.29 ± 4.12	131.04 ± 11.14
Thyme	88.21 ± 1.09	Water	11.97 ± 1.74	18.28 ± 2.16	240.96 ± 3.24	12.88 ± 1.15	32.10 ± 5.21
		Methanol	97.62 ± 10.23	85.51 ± 6.05	1924.83 ± 102.13	154.03 ± 2.99	61.32 ± 12.35
		RMCD	48.05 ± 5.09	7.11 ± 1.27	1523.09 ± 11.27	395.07 ± 13.24	24.18 ± 2.63
		PBS	25.84 ± 1.85	ND	1439.24 ± 19.09	79.92 ± 3.31	4.12 ± 1.14
Basil	88.79 ± 5.72	Water	7.09 ± 1.11	32.77 ± 5.71	336.21 ± 21.01	15.52 ± 2.02	35.41 ± 6.10
		Methanol	211.36 ± 20.71	73.82 ± 5.34	1431.53 ± 35.12	171.00 ± 8.71	72.48 ± 10.04
		RMCD	44.21 ± 5.13	8.27 ± 1.14	1493.21 ± 20.18	301.42 ± 3.79	33.16 ± 2.31
		PBS	38.49 ± 2.35	ND	1532.03 ± 12.08	119.16 ± 11.05	6.41 ± 1.65
Chive	90.10 ± 1.45	Water	1.89 ± 0.18	2.19 ± 0.44	38.81 ± 2.93	26.43 ± 1.90	134.11 ± 8.15
		Methanol	42.23 ± 4.39	4.18 ± 1.09	260.30 ± 10.03	173.06 ± 15.10	228.95 ± 14.03
		RMCD	17.34 ± 1.78	5.42 ± 0.74	153.28 ± 12.72	327.48 ± 3.09	43.62 ± 6.12
		PBS	10.21 ± 0.81	ND	162.21 ± 7.05	128.17 ± 12.05	115.30 ± 8.06

Table 2. TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY ASSAY OF UNBLANCHED FROZEN HERBS (WEEK 1)

Herb	Moisture loss (%)	Extraction solvent	Total phenolic content	Antioxidant activity			
				DPPH	FRAP	CUPRAC	ORAC
Flat leaf parsley	88.82 ± 3.02	Water	1.84 ± 0.21	1.29 ± 0.94	36.59 ± 10.37	26.77 ± 8.12	32.35 ± 9.25
		Methanol	40.66 ± 6.12	2.44 ± 1.50	238.03 ± 11.73	153.94 ± 39.75	126.73 ± 13.75
		RMCD	10.76 ± 0.74	7.89 ± 0.33	1433.64 ± 143.71	219.66 ± 24.73	51.75 ± 15.70
		PBS	22.62 ± 0.55	ND	170.63 ± 20.72	82.66 ±10.03	27.97 ±4.50
Curly leaf parsley	86.82 ± 1.36	Water	1.70 ± 0.88	1.99 ± 1.07	45.41 ± 10.16	14.27 ± 1.75	53.39 ± 12.07
		Methanol	36.47 ± 5.52	2.63 ± 1.36	212.41 ± 21.24	119.71 ± 15.45	134.35 ± 7.50
		RMCD	12.05 ± 2.76	7.28 ± 2.15	1442.73 ± 187.42	170.40 ± 11.06	61.19 ± 23.79
		PBS	13.18 ± 2.09	ND	167.00 ± 14.12	64.41 ± 4.34	39.21 ± 2.40
Corianda	85.71 ± 5.61	Water	5.40 ± 0.95	21.95 ± 3.19	219.84 ± 39.54	14.94 ± 2.08	80.73 ± 15.00
		Methanol	43.94 ± 5.02	6.71 ± 1.80	247.38 ± 25.33	151.98 ± 20.07	149.44 ± 16.16
		RMCD	33.47 ± 1.59	8.15 ± 0.99	1427.87 ± 201.01	185.38 ± 18.15	31.24 ± 12.07
		PBS	37.89 ± 8.11	ND	248.82 ± 24.58	121.12 ± 7.40	130.63 ± 6.04
Mint	88.74 ± 2.05	Water	40.83 ± 9.01	98.59 ± 1.17	1433.15 ± 197.28	13.94 ± 0.77	166.94 ± 14.61
		Methanol	211.53 ± 92.02	90.46 ± 4.79	2412.02 ± 208.87	167.90 ± 12.08	100.42 ± 11.51
		RMCD	92.15 ± 16.02	14.12 ± 1.74	1960.91 ± 21.47	244.63 ± 80.77	33.85 ± 10.21
		PBS	61.08 ± 13.41	ND	1633.64 ± 122.26	127.95 ± 12.38	145.34 ± 20.14
Thyme	91.45 ± 2.11	Water	13.77 ± 1.74	24.02 ± 3.41	247.36 ± 27.24	15.96 ± 0.86	34.91 ± 7.73
		Methanol	101.62 ± 15.23	90.86 ± 7.88	1944.93 ± 93.96	174.83 ± 27.41	72.55 ± 2.77
		RMCD	45.58 ± 9.11	10.23 ± 0.34	1570.00 ± 111.07	408.40 ± 65.02	22.68 ± 8.08
		PBS	20.04 ± 2.08	ND	1479.09 ± 202.09	90.73 ± 10.04	7.17 ± 1.50
Basil	93.53 ± 3.09	Water	17.71 ± 3.46	40.30 ± 2.95	349.52 ± 38.11	20.19 ± 4.13	43.54 ± 7.03
		Methanol	205.13 ± 50.32	88.62 ± 7.73	1491.25 ± 190.77	174.78 ± 15.80	81.04 ± 10.55
		RMCD	70.18 ± 28.43	9.19 ± 0.88	1533.64 ± 53.38	314.28 ± 21.07	38.70 ± 14.34
		PBS	50.43 ± 10.01	ND	1542.73 ± 142.21	124.93 ± 14.08	11.48 ± 5.40
Chive	91.67 ± 2.58	Water	1.78 ± 0.27	6.09 ± 0.87	43.12 ± 8.47	37.10 ± 5.33	140.68 ± 15.17
		Methanol	41.11 ± 8.08	3.48 ± 2.09	269.38 ± 36.46	193.76 ± 16.12	248.25 ± 24.64
		RMCD	22.37 ± 0.78	9.36 ± 2.17	165.18 ± 23.18	331.85 ± 43.09	53.26 ± 11.67
		PBS	11.51 ± 0.77	ND	175.18 ± 20.48	134.27 ± 18.05	121.35 ± 9.10

Table 3. TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY ASSAY OF UNBLANCHED FROZEN HERBS (WEEK 2)

Herb	Moisture loss (%)	Extraction solvent	Total phenolic content	Antioxidant activity			
				DPPH	FRAP	CUPRAC	ORAC
Flat leaf parsley	81.32 ± 6.62	Water	1.42 ± 0.97	3.09 ± 0.99	28.00 ± 3.04	30.01 ± 2.01	37.03 ± 0.54
		Methanol	39.45 ± 2.38	1.73 ± 0.44	188.02 ± 6.10	111.19 ± 12.19	50.02 ± 4.15
		RMCD	2.69 ± 0.93	5.01 ± 1.02	1108.50 ± 24.36	159.76 ± 20.10	40.96 ± 2.12
		PBS	14.27 ± 2.47	ND	167.03 ± 9.46	71.44 ± 3.97	30.14 ± 1.11
Curly leaf parsley	82.01 ± 10.11	Water	2.62 ± 0.06	4.97 ± 0.82	40.51 ± 3.46	22.76 ± 3.44	41.95 ± 2.02
		Methanol	28.09 ± 2.12	2.79 ± 0.46	171.13 ± 8.11	99.31 ± 8.52	110.02 ± 4.17
		RMCD	8.31 ± 1.75	2.93 ± 0.52	1142.01 ± 15.43	152.11 ± 4.29	47.99 ± 6.28
		PBS	15.13 ± 3.03	ND	151.11 ± 13.02	94.56 ± 7.14	35.62 ± 0.85
Corianda	88.20 ± 1.17	Water	4.72 ± 1.27	15.77 ± 3.02	180.12 ± 10.04	22.73 ± 7.29	88.01 ± 4.13
		Methanol	25.88 ± 5.13	6.13 ± 1.08	201.19 ± 7.11	130.11 ± 10.12	137.70 ± 10.18
		RMCD	36.22 ± 7.03	5.74 ± 0.29	1078.59 ± 16.23	189.41 ± 9.13	40.01 ± 2.01
		PBS	16.09 ± 1.56	ND	199.19 ± 12.39	89.01 ± 5.27	98.11 ± 1.22
Mint	90.21 ± 4.08	Water	28.01 ± 3.01	72.03 ± 9.19	1312.10 ± 32.41	22.02 ± 1.15	129.77 ± 3.17
		Methanol	170.20 ± 16.34	84.11 ± 12.05	1978.13 ± 110.01	151.18 ± 5.04	120.16 ± 6.72
		RMCD	85.01 ± 4.13	15.29 ± 1.56	1447.92 ± 92.38	199.06 ± 11.05	31.30 ± 2.12
		PBS	50.11 ± 6.32	ND	1359.77 ± 22.10	118.33 ± 8.34	131.04 ± 11.05
Thyme	85.88 ± 2.01	Water	3.95 ± 0.11	17.45 ± 2.18	216.01 ± 12.02	12.85 ± 1.39	28.10 ± 1.05
		Methanol	98.07 ± 5.03	80.73 ± 5.12	1583.31 ± 52.13	133.51 ± 9.18	60.16 ± 3.02
		RMCD	19.75 ± 2.21	6.81 ± 0.32	1202.74 ± 40.05	323.11 ± 15.31	21.04 ± 2.10
		PBS	18.83 ± 0.38	ND	1120.42 ± 24.12	85.12 ± 8.11	9.52 ± 2.16
Basil	88.11 ± 5.11	Water	15.39 ± 4.07	40.62 ± 2.64	311.72 ± 10.21	33.72 ± 2.14	30.16 ± 3.91
		Methanol	199.33 ± 11.02	82.21 ± 7.13	1132.03 ± 21.09	181.10 ± 5.21	59.92 ± 4.21
		RMCD	38.04 ± 2.47	3.52 ± 0.06	1190.10 ± 102.09	292.67 ± 20.16	30.49 ± 0.77
		PBS	16.79 ± 3.15	ND	1210.27 ± 113.02	112.01 ± 13.04	10.11 ± 2.29
Chive	89.99 ± 6.74	Water	0.92 ± 0.04	3.59 ± 0.81	28.62 ± 3.23	41.02 ± 3.13	119.05 ± 5.23
		Methanol	10.90 ± 1.05	2.27 ± 0.11	129.26 ± 10.03	170.02 ± 10.10	191.02 ± 3.77
		RMCD	13.22 ± 2.07	6.99 ± 1.07	129.43 ± 2.90	236.27 ± 6.05	50.31 ± 6.09
		PBS	5.03 ± 0.28	ND	143.17 ± 9.08	121.95 ± 4.23	101.51 ± 11.02

Table 4. TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY ASSAY OF BLANCHED/FROZEN HERBS (WEEK 1)

Herb	Moisture loss (%)	Extraction solvent	Total phenolic content	Antioxidant activity			
				DPPH	FRAP	CUPRAC	ORAC
Flat leaf parsley	78.15 ± 14.12	Water	1.99 ± 0.57	2.09 ± 1.03	22.61 ± 1.11	22.35 ± 5.07	35.95 ± 2.10
		Methanol	32.16 ± 8.56	1.87 ± 0.96	181.38 ± 10.13	104.68 ± 30.92	45.78 ± 17.15
		RMCD	2.80 ± 1.25	4.21 ± 0.18	1098.00 ± 274.87	163.76 ± 17.83	40.26 ± 9.33
		PBS	18.66 ± 3.01	ND	141.15 ± 12.37	62.34 ± 10.47	23.14 ± 5.11
Curly leaf parsley	82.44 ± 8.57	Water	2.11 ± 0.19	3.12 ± 0.31	33.78 ± 5.24	10.56 ± 3.44	41.44 ± 12.07
		Methanol	24.36 ± 2.38	2.21 ± 0.73	160.67 ± 11.48	87.62 ± 11.72	100.92 ± 27.37
		RMCD	9.02 ± 2.21	3.05 ± 1.72	1138.76 ± 117.56	137.62 ± 9.07	47.83 ± 7.28
		PBS	12.88 ± 6.43	ND	137.58 ± 21.07	91.02 ± 13.54	30.62 ± 11.70
Corianda	89.27 ± 3.09	Water	3.17 ± 0.62	15.05 ± 2.18	167.88 ± 22.53	11.91 ± 3.16	90.01 ± 10.83
		Methanol	29.77 ± 8.14	4.85 ± 1.77	192.11 ± 13.67	100.27 ± 15.08	127.70 ± 45.17
		RMCD	30.19 ± 5.06	3.41 ± 1.92	1098.59 ± 50.03	162.31 ± 20.53	27.99 ± 3.72
		PBS	18.15 ± 2.53	ND	195.64 ± 32.18	89.01 ± 10.07	92.44 ± 18.02
Mint	93.51 ± 4.76	Water	25.11 ± 7.02	70.58 ± 12.09	1120.98 ± 43.78	10.81 ± 3.77	121.87 ± 23.07
		Methanol	159.18 ± 27.34	82.38 ± 5.68	1899.36 ± 140.25	120.21 ± 22.19	117.62 ±14.72
		RMCD	78.69 ± 10.47	10.79 ± 2.15	1405.24 ± 201.61	185.46 ± 31.43	24.37 ± 4.08
		PBS	47.91 ± 10.32	ND	1318.89 ± 93.12	92.12 ± 13.24	121.74 ± 32.05
Thyme	85.38 ± 5.64	Water	7.35 ± 2.11	18.51 ± 3.25	186.15 ± 16.73	9.77 ± 2.51	26.18 ± 7.25
		Methanol	84.01 ± 17.03	83.58 ± 2.47	1561.45 ± 75.45	127.63 ± 18.04	62.96 ± 9.41
		RMCD	28.15 ± 5.21	5.24 ± 1.83	1186.10 ± 92.34	308.05 ± 25.48	17.24 ± 2.10
		PBS	10.99 ± 3.17	ND	1098.15 ± 105.39	69.24 ± 12.59	6.52 ± 0.98
Basil	90.81 ± 2.09	Water	12.43 ± 2.02	47.12 ± 1.92	288.65 ± 43.71	33.29 ± 8.07	32.66 ± 8.01
		Methanol	182.73 ± 20.87	70.28 ± 10.53	1107.79 ± 150.10	163.14 ± 28.01	63.16 ± 7.11
		RMCD	33.58 ± 7.11	3.55 ± 1.09	1138.55 ± 209.05	260.13 ± 17.45	32.41 ± 4.31
		PBS	18.11 ± 5.82	ND	1119.77 ± 183.02	93.20 ± 11.74	8.15 ± 2.83
Chive	88.13 ± 2.58	Water	1.02 ± 0.34	4.18 ± 0.53	31.18 ± 10.21	28.97 ± 5.14	114.10 ± 28.13
		Methanol	14.17 ± 3.18	1.88 ± 0.63	122.26 ± 23.59	149.82 ± 30.12	183.74 ± 35.27
		RMCD	9.44 ± 1.83	6.90 ± 2.47	118.93 ± 12.54	250.61 ± 13.01	48.35 ± 10.72
		PBS	3.37 ± 1.22	ND	133.88 ± 10.78	121.35 ± 05.23	98.59 ± 6.21

Table 5. TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY ASSAY OF BLANCHED/FROZEN HERBS (WEEK 2)

Herb	Moisture loss (%)	Extraction solvent	Total phenolic content	Antioxidant activity			
				DPPH	FRAP	CUPRAC	ORAC
Flat leaf parsley	4.38 ± 1.72	Water	4.61 ± 1.72	74.73 ± 8.05	1438.71 ± 174.66	5.88 ± 1.05	11.29 ± 6.26
		Methanol	87.02 ± 4.89	63.23 ± 10.84	239.00 ± 33.84	31.53 ± 16.70	21.73 ± 13.75
		RMCD	69.27 ± 21.79	17.59 ± 0.24	2940.00 ± 123.45	30.57 ± 14.24	4.84 ± 2.23
		PBS	10.73 ± 1.05	ND	197.91 ± 10.54	12.21 ± 4.07	1.17 ± 0.34
Curly leaf parsley	ND	Water	ND	ND	ND	ND	ND
		Methanol					
		RMCD					
		PBS					
Corianda	3.13 ± 2.04	Water	12.57 ± 0.92	83.15 ± 4.45	5257.25 ± 46.27	2.59 ± 0.12	18.55 ± 8.36
		Methanol	94.98 ± 5.77	61.45 ± 10.06	170.90 ± 13.76	40.37 ± 13.47	6.04 ± 2.42
		RMCD	65.13 ± 3.17	19.24 ± 2.44	3030.91 ± 55.31	35.46 ± 12.98	2.28 ± 0.11
		PBS	18.88 ± 0.48	ND	179.73 ± 1.13	24.06 ± 6.63	1.05 ± 0.42
Mint	6.88 ± 1.26	Water	15.81 ± 3.77	98.96 ± 1.06	3016.70 ± 664.06	2.87 ± 0.64	31.16 ± 14.76
		Methanol	429.27 ± 30.54	84.63 ± 9.62	483.80 ± 26.41	56.43 ± 21.40	33.46 ± 8.08
		RMCD	248.83 ± 41.78	20.10 ± 1.08	5064.55 ± 211.47	37.54 ± 16.43	3.69 ± 1.15
		PBS	52.04 ± 5.12	ND	3849.09 ± 10.74	36.91 ± 11.09	12.48 ± 3.77
Thyme	6.88 ± 0.93	Water	14.86 ± 1.24	88.70 ± 9.27	3661.61 ± 471.49	1.54 ± 0.05	13.18 ± 8.70
		Methanol	295.67 ± 15.28	82.66 ± 11.04	442.36 ± 43.00	49.41 ± 14.21	30.88 ± 8.94
		RMCD	91.62 ± 11.95	22.18 ± 0.74	5473.64 ± 201.83	41.40 ± 7.12	7.63 ± 2.13
		PBS	15.02 ± 0.78	ND	3485.45 ± 11.12	32.80 ± 2.08	8.40 ± 2.40
Basil	6.25 ±2.99	Water	4.66 ± 0.54	83.64 ± 4.50	2185.59 ± 445.46	2.79 ± 0.49	27.33 ± 7.46
		Methanol	403.40 ± 20.80	83.03 ± 7.82	226.45 ± 34.75	40.91 ± 13.56	15.87 ± 5.34
		RMCD	103.14 ± 23.35	20.19 ± 2.42	4982.73 ± 84.75	40.35 ± 10.35	1.07 ± 0.68
		PBS	34.11 ± 1.04	ND	3740.00 ± 31.76	27.62 ± 4.43	9.40 ± 1.83
Chive	6.25 ± 4.16	Water	2.56 ± 0.77	56.93 ± 9.74	925.53 ± 63.17	3.22 ± 0.91	9.09 ± 5.43
		Methanol	60.83 ± 5.63	63.93 ± 12.79	366.47 ± 11.34	34.19 ± 8.33	11.03 ± 3.90
		RMCD	47.36 ± 15.71	18.02 ± 0.28	1824.55 ± 70.99	28.78 ± 11.74	2.10 ± 0.94
		PBS	8.55 ± 0.99	ND	163.36 ± 9.24	22.95 ± 8.14	5.59 ± 0.24

Table 6. TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY ASSAY OF COMMERCIAL DRIED HERBS

Samples	Phytic acid concentration in plants (mg/ g dw ± SD)					
	Fresh potted plants	Dried processed plants	FROZEN HERBS			
			WEEK 1		WEEK 2	
			Fresh Frozen plants	Blanched frozen herbs	Fresh Frozen plants	Blanched frozen herbs
Fl	38.33 ± 2.01	5.88 ± 1.05	28.54 ± 4.01	13.55 ± 1.19	26.77 ± 8.12	10.71 ± 3.51
Cl	13.09 ± 2.50	ND	15.19 ± 1.09	9.01 ± 1.27	14.27 ± 1.75	9.28 ± 2.23
Cr	54.98 ± 7.11	2.59 ± 0.12	18.62 ± 1.32	7.31 ± 0.32	14.94 ± 2.08	3.88 ± 1.09
Mt	54.82 ± 9.24	2.87 ± 0.64	21.04 ± 3.02	8.06 ± 2.05	13.94 ± 0.77	6.27 ± 2.82
Bs	20.78 ± 3.07	1.54 ± 0.05	19.39 ± 4.12	10.02 ± 1.42	20.19 ± 4.13	6.46 ± 1.31
Th	9.14 ± 0.98	2.79 ± 0.48	5.75 ± 0.93	3.76 ± 1.05	5.96 ± 0.86	4.47 ± 0.74
Cv	52.63 ± 11.09	3.22 ± 0.91	41.99 ± 6.10	13.53 ± 2.45	37.10 ± 5.33	8.37 ± 2.85

Table 7. Phytic acid content of herbs. FL (flat leaf parsley), CL (curly leaf parsley), CR (corriander), MT (mint), CV (Chive), BS (Basil), TH (Thyme). All results are mean of triplicate assays.

SAMPLES	FRESH			FROZEN HERBS (WEEK 1)						DRIED/PROCESSED		
				BLANCHED/FROZEN/HERBS			FRESH/FROZEN HERBS					
	TAA (mg/g dw) ± SD	RAA (mg/g dw) ± SD	Oxidized AA (DHA) (mg/g dw)	TAA (mg/g dw) ± SD	RAA (mg/g dw) ± SD	Oxidized AA (DHA) (mg/g dw)	TAA (mg/g dw) ± SD	RAA (mg/g dw) ± SD	Oxidized AA (DHA) (mg/g dw)	TAA (mg/g dw) ±SD	RAA (mg/g dw) ± SD	Oxidized AA (DHA) (mg/g dw)
FL	17.30 ± 2.69	13.25 ± 3.97	4.05±1.28	8.05 ± 1.05	7.23 ± 0.83	0.82±0.22	8.99 ± 1.02	3.91 ± 0.49	5.08±0.53	4.24 ± 1.03	2.75 ± 0.18	1.49
CL	12.93 ± 2.53	11.69 ± 2.02	1.24±0.51	5.71 ± 0.53	5.14 ± 1.13	0.39±0.31	5.03 ± 0.79	4.01 ± 1.37	1.02±0.58	ND	ND	ND
CR	15.59 ± 3.18	12.08 ± 4.54	3.51±1.36	8.11 ± 0.68	6.89 ± 0.95	1.22±0.27	6.31 ± 1.63	3.45 ± 0.09	2.86±1.54	4.86 ± 2.53	2.22 ± 0.75	2.64
MT	25.57 ± 1.22	12.43 ± 1.93	13.14±0.56	14.42 ± 2.09	12.69 ± 0.66	1.73±1.43	8.02 ± 0.48	4.33 ± 0.32	3.69±0.16	3.34 ± 1.08	2.49 ± 0.89	0.85
BS	22.11 ± 0.45	14.43 ± 3.78	7.68±3.33	12.03 ± 1.52	10.83 ± 2.11	1.20±0.59	16.14 ± 3.15	5.07 ± 2.21	11.07±0.94	4.53 ± 1.08	3.11 ± 1.04	1.42
TH	22.83 ± 2.68	17.50 ± 2.92	5.33±0.24	13.28 ± 1.97	11.77 ± 1.24	1.39±0.76	15.98 ± 3.92	6.71 ± 1.13	9.27±2.79	3.94 ± 0.40	2.21 ± 0.80	1.73
CV	22.07 ± 0.99	17.28 ± 1.17	4.79±0.18	9.01 ± 0.57	8.11 ± 0.91	0.90±0.34	10.02 ± 1.81	4.28 ± 1.29	5.74±0.52	3.39 ± 0.91	2.37 ± 0.62	1.02

Table 8: Summary of the total reduced and oxidized ascorbic acid in herbs. FL (flat leaf parsley), CL (curly leaf parsley), CR (coriander), MT (mint), CV (Chive), BS (Basil), TH (Thyme). TAA (total ascorbic acid), RAA (reduced ascorbic acid), DHA (dehydroascorbic acid). All results are mean of triplicate assays.

ND Not determined

SAMPLES	FRESH			FROZEN HERBS (WEEK 2)						DRIED/PROCESSED		
				BLANCHED/FROZEN/HERBS			FESH/FROZEN HERBS					
	TAA (mg/g dw) ± SD	RAA (mg/g dw) ± SD	Oxidized AA (DHA) (mg/g dw)	TAA (mg/g dw) ± SD	RAA (mg/g dw) ± SD	Oxidized AA (DHA) (mg/g dw)	TAA (mg/g dw) ± SD	RAA (mg/g dw) ± SD	Oxidized AA (DHA) (mg/g dw)	TAA (mg/g dw) ±SD	RAA (mg/g dw) ±SD	Oxidized AA (DHA) (mg/g dw)
FL	17.30 ± 2.69	13.25 ± 3.97	4.05±1.28	6.23 ± 0.75	5.78 ± 0.99	0.45±0.24	8.30 ± 3.07	4.82 ± 1.13	3.48±0.51	4.24 ± 1.03	2.75 ± 0.18	1.49
CL	12.93 ± 2.53	11.69 ± 2.02	1.24±0.51	5.21 ± 1.64	4.45 ± 1.02	0.76±0.62	3.28 ± 0.89	2.55 ± 1.45	0.73±0.06	ND	ND	ND
CR	15.59 ± 3.18	12.08 ± 4.54	3.51±1.36	6.08 ± 0.18	6.01 ± 0.52	0.11±0.07	4.55 ± 1.74	2.41 ± 1.23	2.14±0.51	4.86 ± 2.53	2.22 ± 0.75	2.64
MT	25.57 ± 1.22	12.43 ± 1.93	13.14±0.56	13.30 ± 2.96	12.95 ± 0.38	1.84±0.33	7.98 ± 1.88	6.43 ±1.71	1.55±0.17	3.34 ± 1.08	2.49 ± 0.89	0.85
BS	22.11 ± 0.45	14.43 ± 3.78	7.68±3.33	10.83 ± 0.39	10.14 ± 1.74	1.13±0.79	12.45 ± 1.02	8.19 ± 2.04	4.33±0.84	4.53 ± 1.08	3.11 ± 1.04	1.42
TH	22.83 ± 2.68	17.50 ± 2.92	5.33±0.24	10.79 ± 1.01	9.33 ± 0.17	1.44±0.54	9.18 ± 2.63	7.96 ± 2.47	1.22±0.15	3.94 ± 0.40	2.21 ± 0.80	1.73
CV	22.07 ± 0.99	17.28 ± 1.17	4.79±0.18	7.95 ± 0.95	7.12 ± 2.05	1.01±0.21	8.58 ± 1.48	6.23 ± 2.44	2.35±0.11	3.39 ± 0.91	2.37 ± 0.62	1.02

Table 9: Summary of the total reduced and oxidized ascorbic acid in herbs. FL (flat leaf parsley), CL (curly leaf parsley), CR (coriander), MT (mint), CV (Chive), BS (Basil), TH (Thyme). TAA (total ascorbic acid), RAA (reduced ascorbic acid), DHA (dehydroascorbic acid). All results are mean of triplicate assays.

ND Not determined